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ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

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ΕΡΓΑΣΤΗΡΙΟ ΚΛΙΝΙΚΗΣ ΙΟΛΟΓΙΑΣ

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ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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EXPRESSION ANALYSIS OF THE *TP53*, *TP63* AND *TP73* GENES AND THE TAp63, ΔNp63, TAp73 ΚΑΙ ΔNp73 ISOFORMS IN URINARY BLADDER CANCER

ΠΑΠΑΔΟΓΙΑΝΝΗ Γ. ΔΑΝΑΗ

ΒΙΟΛΟΓΟΣ

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«ΑΝΑΛΥΣΗ ΤΩΝ ΜΕΤΑΓΡΑΦΙΚΩΝ ΕΠΙΠΕΔΩΝ ΤΩΝ ΓΟΝΙΔΙΩΝ *TP53*, *TP63* ΚΑΙ *TP73* ΚΑΙ ΤΩΝ ΙΣΟΜΟΡΦΩΝ TAp63, ΔNp63, TAp73 ΚΑΙ ΔNp73 ΣΤΟΝ ΚΑΡΚΙΝΟ ΤΗΣ ΟΥΡΟΔΟΧΟΥ ΚΥΣΤΕΩΣ»

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Στους γονείς μου

Frodo: What are we holding onto, Sam?

Sam: That there's some good in this world, Mr. Frodo... and it's worth fighting for.

(The Lord of the Rings: The Two Towers, written by J. R. R. Tolkien)

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ΕΥΧΑΡΙΣΤΙΕΣ

Οδεύοντας αισίως προς το τέλος της προσπάθειας αυτής, αισθάνομαι την ανάγκη να ευχαριστήσω όσους ανθρώπους με βοήθησαν κατά την διεκπόνηση και ολοκλήρωση της διδακτορικής μου διατριβής.

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δικαιωματικά αφιερωμένη σε αυτούς, στον παππού μου Γιώργο και την γιαγιά μου Γεωργία και στην μνήμη του παππού μου Στέλιου και της γιαγιάς μου Δανάης. Ο καθένας με τον δικό του τρόπο, αλλά και όλοι τους μαζί, μου δίδαξαν την αξία της ανιδιοτελούς αγάπης και του δίκαιου και έντιμου αγώνα για το καλό που υπάρχει σ' αυτό τον κόσμο.

ABSTRACT

The urinary bladder is a musculomembranous subperitoneal organ which functions as a reservoir for the urine in the human body. Urinary bladder cancer is the 11th most common malignancy worldwide and the 4th most common among males in the United States, where 74,690 new cases will be diagnosed in 2014 alone. Smoking and occupational exposure to aromatic amines are recognized as major risk factors for the development of the disease, followed by chronic urinary tract infection, Schistosoma infection in certain geographical regions, exposure to cyclophosphamide and radiotherapy to adjacent organs. It is estimated that 90-95% of bladder cancer cases are urothelial carcinomas, whereas the remaining 5-10% are mostly squamous cell carcinomas and adenocarcinomas. Determination of tumor stage at diagnosis is crucial for prognosis and patient management, as two major phenotypic variants of urothelial tumors with drastically different biological behavior have been recognized. Low-grade non-muscle-invasive tumors, comprising approximately 80% of the cases, have limited invasive potential and come with favorable prognosis when treated early, although accompanied by high recurrence rates. On the contrary, high-grade muscle-invasive tumors have very bad prognosis, with most of the patients developing life-threatening metastases despite surgical resection and systemic therapy. The genetic alterations underlying the development of urothelial cancer include chromosomal aberrations, inactivating tumor-suppressor gene mutations and activation of oncogenes. FGFR3 mutations are detected in more than 70% of non-muscle-invasive tumors, whereas p53 is mutated in almost 50% of the muscle-invasive lesions.

TP53 is the most extensively studied tumor-suppressor gene. Mutations in the *TP53* gene have been reported in the majority of human malignancies, suggesting a significant contribution to the development and progression of human tumors. Two *TP53* homologs, *TP63* and *TP73*, have recently been identified. Though highly similar with p53 regarding genomic organization, both p63 and p73 utilize complex mechanisms to provide two major classes of protein isoforms with tumor-suppresive (TA-isoforms) or tumorigenic function (ΔN-isoforms). TAp63 and TAp73 exhibit p53-like function in terms of inducing apoptosis, whereas the N-terminally truncated ΔNp63

and ΔNp73 act as dominant negative inhibitors of both wild-type p53 protein and the respective TA-isoforms. Both *TP63* and *TP73* are rarely mutated in human tumors, whereas their deletion from mice results in various developmental and neurological abnormalities rather than the development of malignant lesions. Overall, it is evident that the two *TP53* homologs are not to be considered as classic tumor-suppressor genes.

The objective of this PhD thesis was to determine the expression levels of the three p53 family genes (*TP53*, *TP63*, *TP73*) and the *TP63* and *TP73* TA and ΔN isoforms in bladder tumors and to evaluate any alterations in their expression patterns that might be associated with the development and progression of urothelial malignancies. The expression analysis was performed in a set of 30 urothelial tumors, matched with normal tissues, which were obtained from bladder cancer patients who underwent surgical resection of bladder tumor.

Expression analysis revealed elevated p53, p63 and p73 mRNA levels in 47.0%, 40.0% and 43.0% of the samples, respectively. p53, p63 and p73 exhibited a 1.9-fold, 3.0-fold and 2.0-fold overexpression in tumor specimens compared with the normal samples, respectively. In the normal bladder epithelium, all members of the p53 gene family were co-expressed ($p < 0.001$). However, in malignant urothelium, only the positive co-expression between p63 and p73 was maintained ($p=0.008$). Statistical analysis revealed that p53 was up-regulated in non-muscle-invasive (pTa-T1) tumors compared with muscle-invasive (pT2-T4) ones ($p=0.047$). Significantly higher p63 mRNA levels were measured in malignant samples from patients > 75 years of age versus younger patients ($p=0.022$) and from patients with disease relapse compared with cancer-free patients ($p=0.045$). p63 expression significantly deviated from the normal pattern in papillary versus non-papillary lesions ($p=0.026$). p73 overexpression was observed in Grade III tumors compared with Grade II tumors, in patients presenting with one-site lesions ($p=0.040$).

Also, elevated TAp63, ΔNp63, TAp73 and ΔNp73 mRNA levels were measured in 33.0%, 50.0%, 40.0% and 37.0% of the samples, respectively. TAp63, ΔNp63, TAp73 and ΔNp73 exhibited a 2.9-fold, 3.2-fold, 1.7-fold and 3.3-fold overexpression in tumor

specimens compared with the normal samples, respectively. Tumor-specific TAp63 expression was detected in 3 cases, while TAp63 was expressed only in normal tissue in 4 cases. Tumor-specific ΔNp73 expression was detected in 7 cases, while ΔNp73 was expressed only in normal tissue in 4 cases. ΔNp73 was also not expressed in 3 tissue pairs. The ΔNp63/TAp63 isoform ratio shifted 1.1-fold in favor of ΔNp63, and the ΔNp73/TAp73 isoform ratio shifted 2.0-fold in favor of ΔNp73 from normal to tumor specimens. In normal urothelium TAp73 was co-expressed with ΔNp73 (p=0.003). In the malignant bladder epithelium ΔNp63 negatively correlated with TAp73 (p=0.006). Statistical analysis revealed that ΔNp63 was up-regulated in tumor samples from bladder cancer patients > 75 years old versus younger ones (p=0.026), in non-muscle-invasive (pTa-T1) tumors versus muscle-invasive ones (pT2-T4) (p=0.019) and in patients who received BCG treatment versus those who were treated with cystectomy (p=0.045). TAp73 mRNA levels were increased in patients with papillary tumors compared with those with non-papillary ones (p=0.006) and in current smokers compared with non-smokers and ex-smokers combined (p=0.020).

Our results indicate that expression of all *TP53* family members deviate from the normal pattern in bladder tumors. p63 overexpression is associated with a less aggressive tumor phenotype, whereas p73 up-regulation correlates with an unfavorable patient outcome. We also provide evidence that p63 and p73 exert their contribution to the malignant transformation of the bladder epithelium independently of p53. In addition, our results support an anti-invasive role for ΔNp63 in urothelial cancers and a potentially protective effect of TAp73 on bladder epithelium. Finally, we provide evidence for epigenetic regulation of p63 and p73 isoforms expression in normal and malignant urothelium.

ΠΕΡΙΛΗΨΗ

Η ουροδόχος κύστη είναι ένα μυομεμβρανώδες υποπεριτοναϊκό όργανο, το οποίο λειτουργεί ως δεξαμενή για τα ούρα στο ανθρώπινο σώμα. Ο καρκίνος της ουροδόχου κύστεως είναι η 11^{η} πιο συχνή μορφή κακοήθειας στον κόσμο και η 4^{η} μεταξύ των ανδρών στις ΗΠΑ, όπου μόνο το 2014 θα διαγνωσθούν 74.690 νέα περιστατικά της νόσου. Το κάπνισμα και η έκθεση στις αρωματικές αμίνες έχουν αναγνωρισθεί ως κύριοι παράγοντες κινδύνου για την εμφάνιση της νόσου, ενώ ακολουθούν οι χρόνιες λοιμώξεις της ουροποιητικής οδού, η μόλυνση από το παράσιτο Schistosoma σε συγκεκριμένες γεωγραφικές περιοχές, η έκθεση στην κυκλοφωσφαμίδη και η ακτινοθεραπεία σε παρακείμενα όργανα. Υπολογίζεται ότι το 90-95% των όγκων είναι ουροθηλιακά καρκινώματα, ενώ το υπόλοιπο 5-10% είναι ως επί το πλείστον καρκινώματα εκ πλακώδους επιθηλίου και αδενοκαρκινώματα. Ο καθορισμός του παθολογικού σταδίου του όγκου κατά την διάγνωση είναι καθοριστικής σημασίας για την πρόγνωση, καθώς έχουν αναγνωρισθεί δύο φαινοτυπικές μορφές της νόσου με ριζικά διαφορετική βιολογική συμπεριφορά. Οι καλά διαφοροποιημένοι (χαμηλού grade), μη μυοδιηθητικοί όγκοι αποτελούν το 80% των περιστατικών, έχουν περιορισμένο μεταστατικό δυναμικό και ευνοϊκή πρόγνωση όταν αντιμετωπιστούν έγκαιρα, αν και συνοδεύονται από υψηλά ποσοστά υποτροπών. Αντίθετα, οι φτωχά διαφοροποιημένοι (υψηλού grade) μυοδιηθητικοί όγκοι έχουν πολύ κακή πρόγνωση, με τους περισσότερους ασθενείς να εμφανίζουν θανάσιμες μεταστάσεις παρά την χειρουργική αφαίρεση και την συστημική θεραπεία. Οι γενετικές μεταβολές που υπόκεινται στην εμφάνιση της νόσου περιλαμβάνουν χρωμοσωμικές ανωμαλίες, μεταλλάξεις που απενεργοποιούν ογκοκατασταλτικά γονίδια και ενεργοποίηση ογκογονιδίων. Μεταλλάξεις στον FGFR3 ανιχνεύονται σε περισσότερους από το 70% των μη μυοδιηθητικών όγκων, ενώ το p53 βρίσκεται μεταλλαγμένο σε περίπου 50% των μυοδιηθητικών όγκων.

Το *TP53* είναι το καλύτερα μελετημένο ογκοκατασταλτικό γονίδιο. Μεταλλάξεις στο γονίδιο *TP53* έχουν αναφερθεί στην πλειοψηφία των ανθρώπινων όγκων, γεγονός που υποδεικνύει σημαντική συνεισφορά του γονιδίου αυτού στην εμφάνιση κακοηθειών. Πρόσφατα, αναγνωρίσθηκαν τα ομόλογα γονίδια του *TP53*, τα *TP63* και

TP73. Αν και εμφανίζουν σημαντικές ομοιότητες με το *TP53* όσον αφορά την γονιδιακή οργάνωση, τα *TP63* και *TP73* χρησιμοποιούν περίπλοκους μηχανισμούς προκειμένου να παράξουν δύο κύριες κατηγορίες πρωτεϊνικών ισομορφών με ογκοκατασταλτική (ΤΑ ισομορφές) ή με ογκογόνο δράση (ΔΝ ισομορφές). Οι ισομορφές TAp63 και TAp73 έχουν δράση παρόμοια με την p53 όσον αφορά την επαγωγή της απόπτωσης, ενώ οι ΔNp63 και ΔNp73 (από τις οποίες λείπει το αμινοτελικό άκρο) ρυθμίζουν με αρνητικό επικρατή τρόπο την αγρίου τύπου p53 πρωτεΐνη, αλλά και τις αντίστοιχες ΤΑ ισομορφές. Μεταλλάξεις στα *TP63* και *TP73* σπάνια ανιχνεύονται στους ανθρώπινους όγκους, ενώ η απαλειφή τους από πειραματόζωα έχει ως αποτέλεσμα την εμφάνιση σοβαρότατων αναπτυξιακών και νευρολογικών ανωμαλιών παρά την ανάπτυξη καρκινικών όγκων. Είναι λοιπόν προφανές ότι τα *TP63* και *TP73* δεν μπορεί να θεωρούνται κλασικά ογκοκατασταλτικά γονίδια.

Σκοπός της παρούσας διδακτορικής διατριβής ήταν ο προσδιορισμός των μεταγραφικών επιπέδων των τριών γονιδίων της οικογένειας p53 (*TP53*, *TP63*, *TP73*) και των ΤΑ και ΔΝ ισομορφών των γονιδίων *TP63* και *TP73* και η αξιολόγηση πιθανών μεταβολών στα πρότυπα έκφρασής τους οι οποίες θα μπορούσαν να συσχετισθούν με την εμφάνιση και την εξέλιξη της νόσου. Η ανάλυση της έκφρασης πραγματοποιήθηκε σε 30 ουροθηλιακούς καρκίνους, και 30 αντίστοιχους φυσιολογικούς ιστούς, οι οποίοι ελήφθησαν από ασθενείς με καρκίνο της ουροδόχου κύστεως που υπεβλήθησαν σε χειρουργική αφαίρεση του όγκου.

Η ανάλυση έδειξε αυξημένα μεταγραφικά επίπεδα των p53, p63 and p73 στο 47,0%, 40,0% και 43,0% των δειγμάτων, αντίστοιχα. Υπερέκφραση των p53, p63 και p73 κατά 1,9, 3,0 και 2,0 φορές αντίστοιχα ανιχνεύθηκε στα καρκινικά δείγματα σε σύγκριση με τα φυσιολογικά. Στο φυσιολογικό ουροθήλιο, όλα τα γονίδια της οικογένειας p53 συνεκφράστηκαν ($p < 0,001$). Όμως, στο καρκινικό ουροθήλιο, διατηρήθηκε μόνο η συνέκφραση των p63 και p73 ($p=0,008$). Η στατιστική ανάλυση έδειξε ότι το p53 υπερκφράζεται στους μη μυοδιηθητικούς όγκους (παθολογικών σταδίων Τα-Τ1) σε σύγκριση με τους μυοδιηθητικούς (παθολογικών σταδίων Τ2-Τ4) ($p=0,047$). Σημαντικά υψηλότερα μεταγραφικά επίπεδα του p63 μετρήθηκαν σε καρκινικά δείγματα από ασθενείς άνω των 75 ετών σε σύγκριση με αυτά από νεότερους ($p=0,022$) και από

ασθενείς με υποτροπή της νόσου σε σύγκριση με αυτά από ασθενείς χωρίς υποτροπή (p=0,045). Η έκφραση του p63 βρέθηκε να αποκλίνει σημαντικά από το φυσιολογικό πρότυπο στους θηλώδεις όγκους σε σύγκριση με τους μη θηλώδεις (p=0,026). Παρατηρήθηκε υπερέκφραση του p73 στους υψηλού grade (grade III) όγκους σε σύγκριση με τους grade II όγκους, σε ασθενείς που διαγνώσθηκαν με μονήρεις όγκους (p=0,040).

Επίσης, αυξημένα μεταγραφικά επίπεδα των ισομορφών TAp63, ΔNp63, TAp73 και ΔNp73 μετρήθηκαν σε 33,0%, 50,0%, 40,0% και 37,0% των δειγμάτων, αντίστοιχα. Υπερέκφραση των TAp63, ΔNp63, TAp73 και ΔNp73 κατά 2,9, 3,2, 1,7 και 3,3 φορές αντίστοιχα ανιχνεύθηκε στα καρκινικά δείγματα σε σύγκριση με τα φυσιολογικά. Μετάγραφα της ισομορφής TAp63 ανιχνεύθηκαν μόνο στο καρκινικό δείγμα σε 3 περιπτώσεις, ενώ τα ίδια μετάγραφα ανιχνεύθηκαν μόνο στο φυσιολογικό δείγμα σε 4 περιπτώσεις. Μετάγραφα της ισομορφής ΔNp73 ανιχνεύθηκαν μόνο στο καρκινικό δείγμα σε 7 περιπτώσεις, ενώ τα ίδια μετάγραφα ανιχνεύθηκαν μόνο στο φυσιολογικό δείγμα σε 4 περιπτώσεις. Επίσης, μετάγραφα της ισομορφής ΔNp73 δεν ανιχνεύθηκαν σε 3 ζεύγη ιστών. Ο λόγος έκφρασης ΔNp63/TAp63 αυξήθηκε κατά 1,1 φορά υπέρ της ισομορφής ΔNp63, και ο αντίστοιχος λόγος ΔNp73/TAp73 αυξήθηκε κατά 2,0 φορές υπέρ της ισομορφής ΔNp73 από τα φυσιολογικά στα καρκινικά δείγματα. Στο φυσιολογικό ουροθήλιο, οι ισομορφές TAp73 και ΔNp73 συνεκφράστηκαν (p=0,003). Στο καρκινικό ουροθήλιο, η έκφραση των ισομορφών ΔNp63 και TAp73 εμφάνισε αρνητική συσχέτιση (p=0,006). Η στατιστική ανάλυση έδειξε ότι η ισομορφή ΔNp63 υπερεκφράζεται στα καρκινικά δείγματα από ασθενείς άνω των 75 ετών σε σύγκριση με αυτά από νεότερους (p=0,026), στους μη μυοδιηθητικούς όγκους (παθολογικών σταδίων Ta-T1) σε σύγκριση με τους μυοδιηθητικούς (παθολογικών σταδίων T2-T4) (p=0,019) και σε ασθενείς που υπεβλήθησαν σε θεραπεία BCG σε σύγκριση με αυτούς που υπεβλήθησαν σε κυστεκτομή (p=0,045). Σημαντικά υψηλότερα μεταγραφικά επίπεδα της ισομορφής TAp73 μετρήθηκαν σε ασθενείς με θηλώδεις όγκους σε σύγκριση με τους ασθενείς με μη θηλώδεις όγκους (p=0,006) και σε καπνιστές σε σύγκριση με τους μη καπνιστές και τους πρώην καπνιστές αθροιστικά (p=0,020).

Τα αποτελέσματά μας δείχνουν ότι η έκφραση και των τριών γονιδίων της οικογένειας p53 αποκλίνει από το φυσιολογικό πρότυπο στους όγκους της ουροδόχου κύστεως. Η υπερέκφραση του p63 συσχετίσθηκε με έναν λιγότερο επιθετικό φαινότυπο, ενώ η υπερέκφραση του p73 συσχετίσθηκε με μη ευνοϊκή πρόγνωση. Επίσης, παρέχονται ενδείξεις ότι τα p63 and p73 συμβάλλουν στον κακοήθη μετασχηματισμό του ουροθηλίου ανεξάρτητα από το p53. Επίσης, τα αποτελέσματά μας υποστηρίζουν τον αντι-διηθητικό ρόλο που έχει προταθεί για την ισομορφή ΔNp63 στους ουροθηλιακούς καρκίνους, αλλά και μία πιθανόν προστατευτική επίδραση της ισομορφής TAp73 στο ουροθήλιο. Τέλος, παρέχονται ενδείξεις για επιγενετική ρύθμιση της έκφρασης των ισομορφών των p63 and p73 στο φυσιολογικό και το καρκινικό ουροθήλιο.

1. THE URINARY BLADDER

1.1. The urinary system – Overview of parts and functions

The urinary system in humans consists of the kidneys, the ureters, the bladder and the urethra (Figure 1.1.1). The kidneys and the ureters are two in number, whereas the urethra is different in males and females. The urinary system is responsible for:

- formation and emptying of urine -along with waste products of the metabolism, like urea and uric acid- out of the human body,
- regulation of water balance and
- regulation of electrolyte (e.g. sodium and potassium) balance [1].

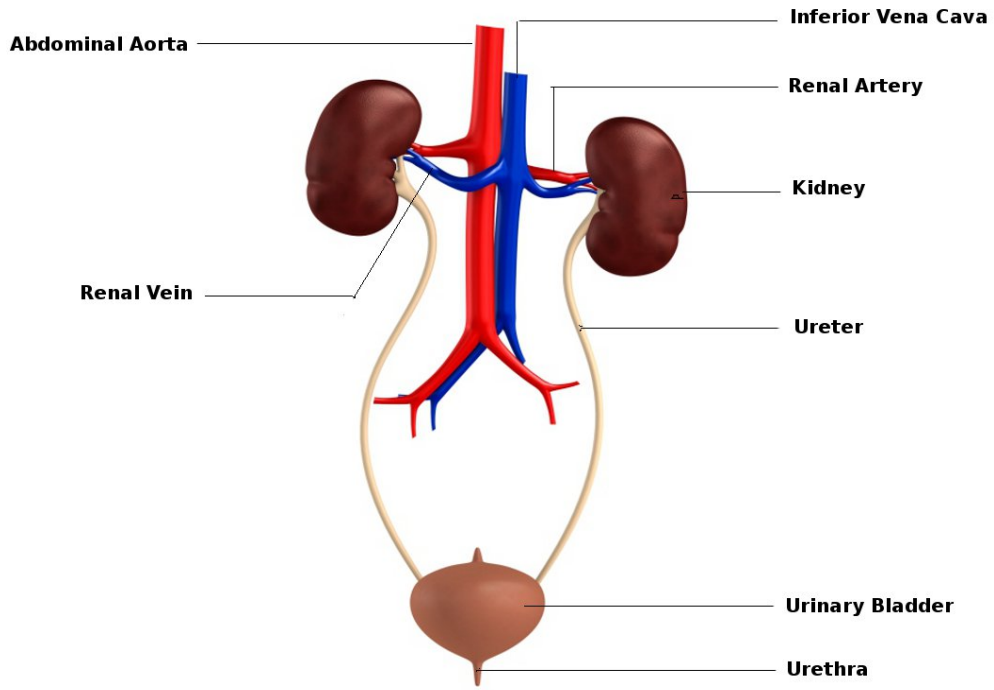


Figure 1.1.1: The human urinary system.

1.2. The kidneys

1.2.1. Structure of the kidneys

The kidneys are located in the posterior part of the abdominal cavity and behind the peritoneum, one on either side of the spine, and are surrounded by fat and areolar tissue. Their upper extremities are on a level with the upper border of the twelfth thoracic vertebra and their lower extremities are on a level with the third lumbar. The right kidney is usually located slightly lower than the left, presumably due to the presence of the liver above it. Each kidney is approximately 11.25 cm in length, 5 to 7.5 cm in breadth, and more than 2.5 cm in thickness. The weight of the kidney in the adult male varies from 125 to 170 g, in the adult female from 115 to 155 g, whereas the combined weight of the two kidneys in proportion to that of the body is about 1 to 240. The kidney has a characteristic pear-shaped form and presents (i) the anterior and posterior surfaces, (ii) the lateral and medial border and (iii) the superior and inferior extremities [1].

The medial side of each kidney forms an indentation through which pass the renal artery and vein, nerves, and pelvis. Cross-section reveals two regions in the kidney: an outer region called the cortex and an inner region called the medulla**.** The cortex and medulla consist of the nephrons, which are the functional units of the kidney, as well as of blood vessels, lymphatics, and nerves. The medulla consists of striated conical masses of red color, called the renal pyramids. The base of each pyramid is directed toward the circumference of the kidney and its apex terminates in a papilla. The pelvis is the upper expansion of the ureter**,** which is in turn responsible for carrying urine from the pelvis to the urinary bladder. In the human kidney, three open-ended pouches, called the major calyces, form a funnel-shaped sac, termed the renal pelvis. Each major calyx is subdivided in numerous minor calyces, whose role is to collect urine from each papilla (Figure 1.2.1). The walls of the calyces, pelvis and ureters are invested with smooth muscle which contracts to propel the urine towards the bladder [1, 2].

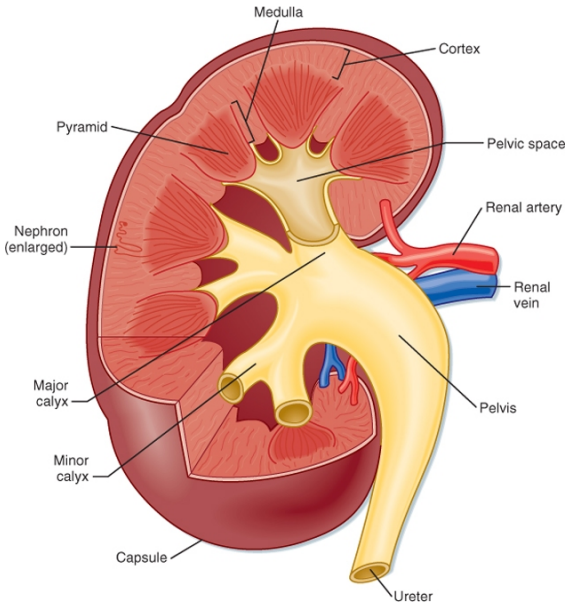


Figure 1.2.1: Gross anatomic features of the human kidney. (Modified from Marsh DJ: Renal Physiology, New York 1983, Raven Press)

1.2.2. Ultrastructure of the nephron – Urine production

The nephron is the functional unit of the kidneys, with each human kidney containing approximately 1.2 million of these tubular structures. The nephrons consist of the renal corpuscle, the proximal tubule, the loop of Henle, the distal tubule and the collecting duct system. The renal corpuscle consists of glomerular capillaries and of Bowman's capsule. The proximal tubule initially forms coil-shaped structures, and then presents as a straight piece descending towards the medulla. The loop of Henle consists of the straight part of the proximal tubule, the descending thin limb (which ends in a hairpin turn), the ascending thin limb (only in nephrons with long loops of Henle), and the thick ascending limb. Near the end of the thick ascending limb (termed the macula densa), the nephron passes between the afferent and efferent arterioles. The distal tubule begins shortly beyond the macula densa and extends to the point in the cortex where two or more nephrons join to form a cortical collecting duct. The cortical collecting duct enters the medulla, where it continues as the outer medullary collecting duct and the inner medullary collecting duct (Figure 1.2.2) [1, 2].

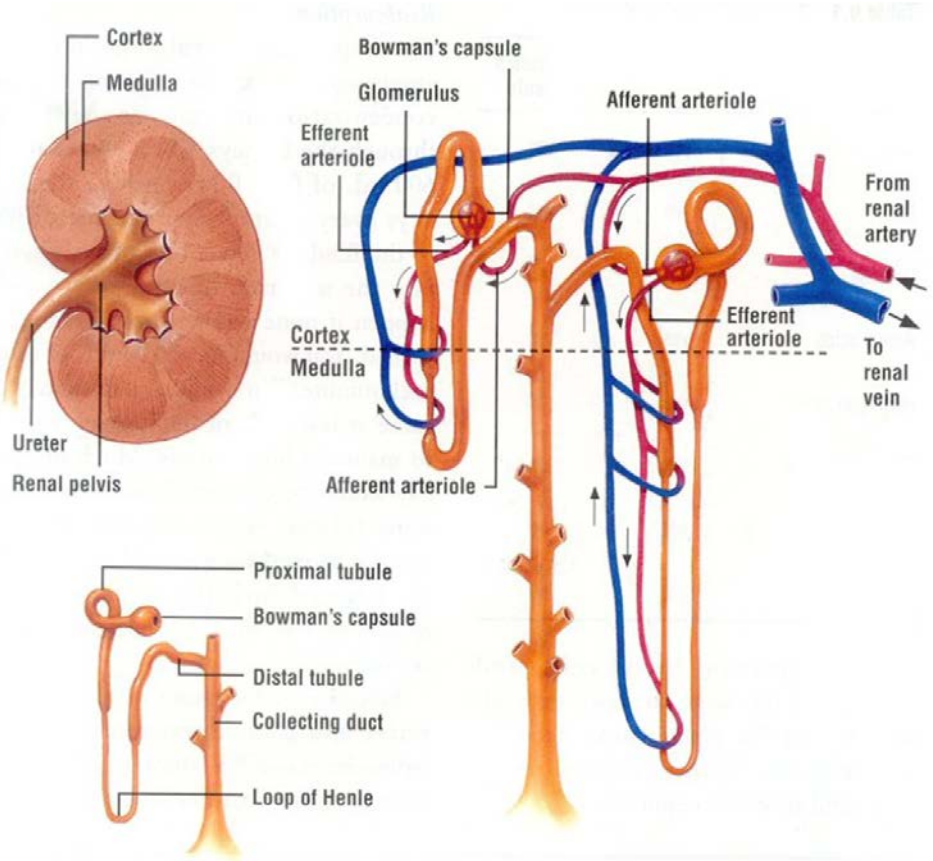


Figure 1.2.2: Organization of the human nephron.

1.2.3. Urine formation

Urine formation involves three processes: glomerular filtration, tubular reabsorption and tubular secretion (Figure 1.2.3).

In the first step, blood infiltrates the walls of the glomerular capillaries into Bowman's space, resulting in the formation of the glomerular ultrafiltrate, a fluid composed mostly of water and the same solutes present in blood plasma, with the exception of proteins (Figure 1.2.4). The main force that contributes to the molecules' crossing the capillary walls is the hydrostatic pressure of blood entering the corpuscle [2, 3].

Subsequently, substances such as glucose, amino acids and salts are returned by diffusion and active transport out of the tubular fluid, through the epithelium of the renal tubule, into the interstitial fluid and the peritubular capillaries and finally back

into the blood stream. Reabsorption of water and sodium also takes place by osmosis or active transport respectively [2, 3].

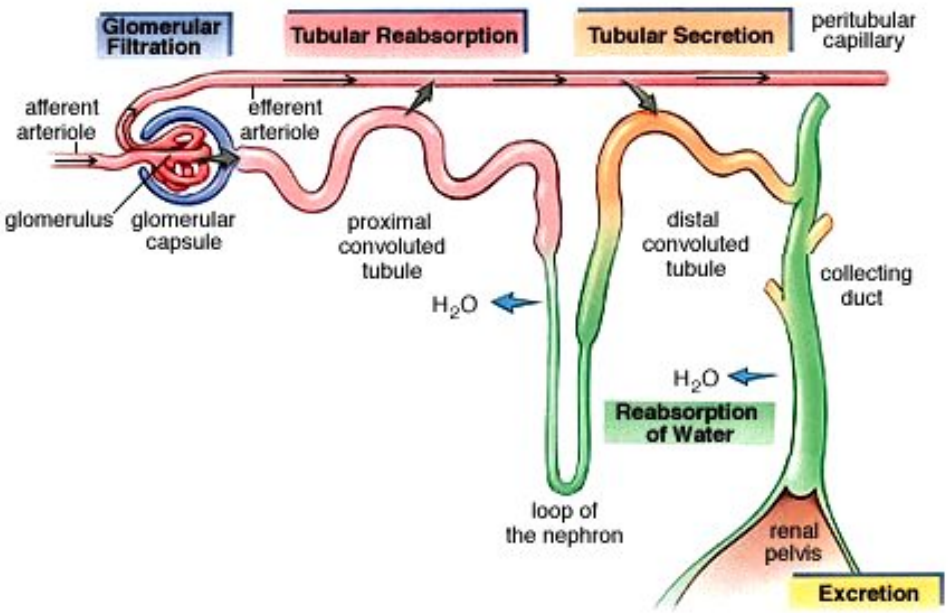


Figure 1.2.3: Overview of the processes of urine formation. (© The McGraw-Hill Companies, Inc.)

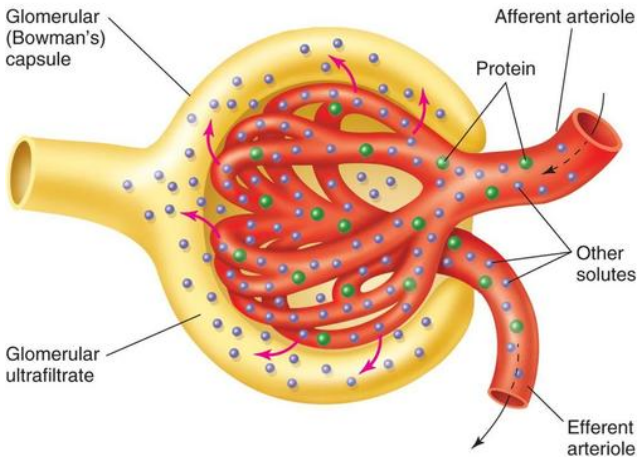


Figure 1.2.4: Glomerular filtration. (© The McGraw-Hill Companies, Inc.)

Last, during tubular secretion molecules such as ammonia, hydrogen ions and other substances are actively transported from the blood into the proximal and distal convoluted tubules and finally to the collecting duct, where additional water is reabsorbed [2, 3].

After forming in the nephrons, urine passes to the renal papillae and the calyces. Of them, the major calyces feed into the renal pelvis, from which the urine is directed to the ureters and the urinary bladder. The renal calyces, the pelvis, the ureters and the urinary bladder are invested with transitional epithelium, also termed urothelium. The transitional epithelium is multi-layered and can adapt to volume changes by contraction and expansion [2, 3].

1.3. The ureters

The ureters are two narrow, cylindrical tubes that measure approximately 30cm in length. They commence from the renal pelvis, from which they carry urine to the urinary bladder. Structurally, they compose of the fibrous, muscular and mucous coats. The muscle tissue is found only in the neighborhood of the bladder and assists to conveying urine into it by peristaltic movements. The mucous coat is smooth and of transitional character [1].

1.4. The urinary bladder

The urinary bladder is a musculomembranous subperitoneal organ which functions as a reservoir for the urine in the human body. Its size, position and relations are variable and depend on the amount of fluid it contains. When empty, it lies in the lesser pelvis; as it distends, it expands anterosuperiorly into the abdominal cavity [1, 4].

The bladder contains a fundus (base), a neck, an apex, a superior and two inferolateral surfaces. When hardened *in situ* and devoid of urine, the bladder looks like a flattened tetrahedron, with its apex tilted forward. The fundus is triangular and is located posteroinferiorly towards the rectum, from which it is separated by the rectovesical

fascia, the seminal vesicles and the terminal portions of the ductus deferentes in males. In females, it is closely related to the anterior vaginal wall. The bladder neck, which essentially comprises the internal urethral orifice, is directed inferiorly, 3–4 cm behind the lower part of the symphysis pubis and just above the plane of the inferior aperture of the lesser pelvis. Its position is constant and independent of the varying positions of the bladder and rectum. In males the neck rests on, and is in direct continuity with, the base of the prostate; in females it is related to the pelvic fascia, which surrounds the upper urethra.

In both sexes, the apex is directed forward towards the upper part of the symphysis pubis and from it the middle umbilical ligament ascends to the umbilicus. The anterior surface of the bladder is separated from the transversalis fascia by fatty tissue. The inferolateral surfaces are not covered by peritoneum and are related anteriorly to the pubis and puboprostatic or pubovesical ligaments in males and females respectively. The superior surface is triangular, directed upward and covered by peritoneum. It is bounded by lateral borders from the apex to the ureteric entrances and by a posterior border which joins them. In males, the superior surface extends slightly onto the base and continues posteriorly into the retrovesical pouch and anteriorly into the median umbilical fold; it is also in contact with the sigmoid colon and some of the coils of the small intestine. When the bladder is empty and contracted, the superior surface is convex and upon it rests the uterus in females. When the bladder is relaxed, the superior surface is concave and its interior appears as a V-shaped slit (in a median sagittal section), in which the apex of the V corresponds to the internal orifice of the urethra. The internal urethral orifice along with the orifices of the ureters form the trigone of the urinary bladder. The inferior surface is directed downward and is uncovered by peritoneum. It can be divided into a posterior or prostatic area and two inferolateral surfaces. The posterior area, from which the urethra emerges, is in direct continuity with the base of the prostate in males. The inferolateral surfaces are directed downward and laterally. In front, they are separated from the symphysis pubis by a mass of fatty tissue, the retropubic pad; behind, they are in contact with the fascia which covers the levator ani and obturator internus muscles. In females, the bladder is connected by areolar tissue to the front of the cervix uteri and the upper

part of the anterior wall of the vagina, whereas it is separated from the anterior surface of the body of the uterus by the vesicouterine pouch (Figures 1.3.1, 1.3.2) [1, 4].

Structurally, the bladder is composed of the serous, muscular, submucous and mucous coats [1].

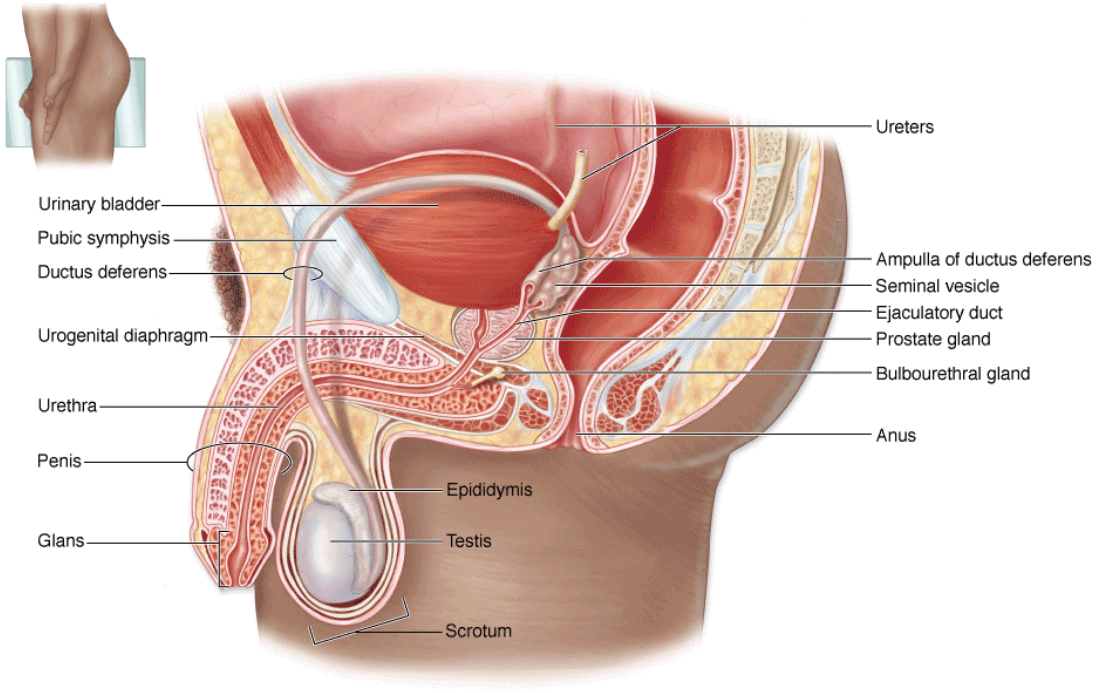


Figure 1.3.1: Relations of the male urinary bladder as seen in sagittal section of the pelvis. (© The McGraw-Hill Companies, Inc.)

The serous coat is the outer layer that derives from the peritoneum and invests the superior surface and the upper parts of the lateral surfaces. The muscular coat (muscularis propria) consists of an internal, a middle and an external layer of muscle fibers. Two bands of oblique fibers of the internal layer form the muscles of the ureters, whereas the sphincter muscle of the bladder is formed by fibers of the middle layer. The detrusor urinae muscle consists of fibers of the external layer. The submucous coat (lamina propria) consists of areolar tissue and connects the muscular

and mucous coats. The mucous coat is a thin and smooth layer covered by transitional epithelium [1].

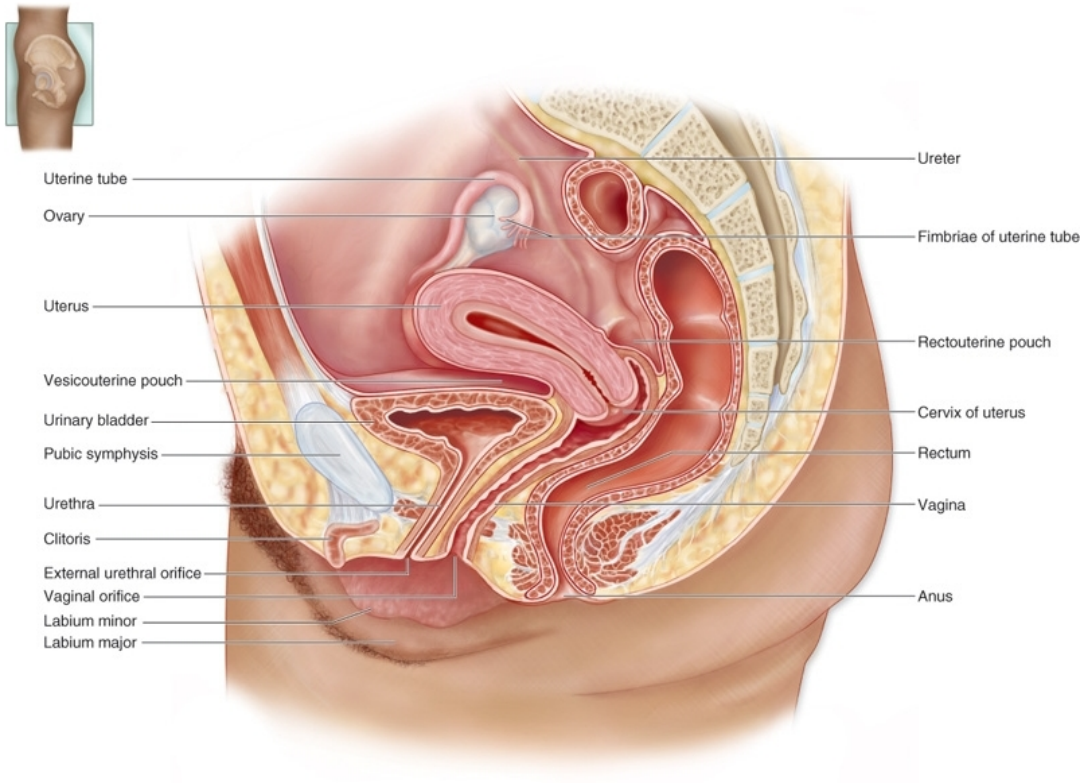


Figure 1.3.2: Relations of the female urinary bladder as seen in sagittal section of the pelvis. (© The McGraw-Hill Companies, Inc.)

1.4.1. Innervation of the urinary bladder

The innervation of the urinary bladder and the urethra plays a central role in controlling urination. The smooth muscle fibers of the bladder neck are innervated by the hypogastric nerves. Alpha-adrenergic receptors are located mainly in the bladder neck and the urethra and can cause contraction and induce closure of the urethra upon stimulation, thus facilitating the storage of urine. The body of the bladder receives parasympathetic innervation by sacral nerves, which can cause a sustained bladder contraction via stimulation of muscarinic receptors. The fundus of the bladder is innervated by sensory fibers of the pelvic nerves; these sensory fibers carry input

from receptors that detect bladder fullness, pain, and temperature sensation. The skeletal muscle fibers of the external sphincter are controlled by the sacral pudendal nerves, which can cause contraction by producing excitatory impulses (Figure 1.4.1) [2].

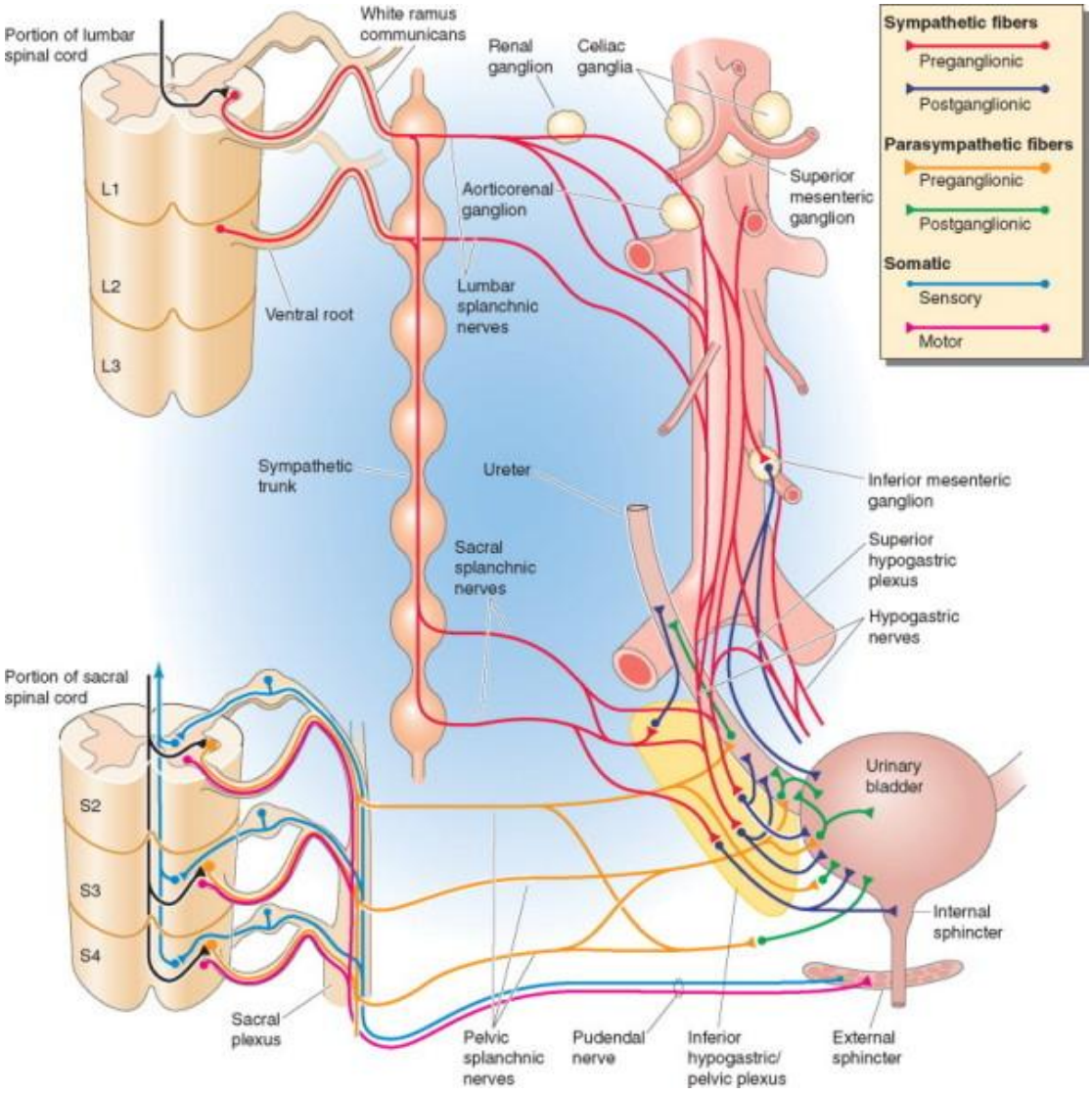


Figure 1.4.1: Innervation of the urinary bladder.

1.5. The urethra

The urethra is a duct that facilitates the disposal of urine from the bladder and out of the body. It is anatomically linked with the reproductive structures in males, whereas in females the urethra is exclusively part of the urinary system.

1.5.1. The male urethra

The male urethra measures from 17.5 to 20 cm in length and extends from the internal urethral orifice in the urinary bladder to the external urethral orifice at the end of the penis. It serves as an exit for urine as well as for semen out of the male body and is divided into the prostatic, the membranous and the cavernous (or spongy) portions. Except during the passage of the urine or semen, the greater part of the urethral canal is a transverse slit, with its upper and under surfaces in contact; at the external orifice the slit is vertical, in the membranous portion irregular or stellate, and in the prostatic portion somewhat arched (Figure 1.5.1) [1].

1.5.2. The female urethra

The female urethra measures about 4 cm in length and extends from the internal to the external urethral orifice. It is located behind the symphysis pubis, embedded in the anterior wall of the vagina, with direction obliquely downward and forward. Its external orifice is located directly in front of the vaginal opening and about 2.5 cm behind the clitoris. Between the superior and inferior fasciae of the urogenital diaphragm, the female urethra is surrounded by the sphincter urethral muscle, as in the male (Figure 1.5.2) [1].

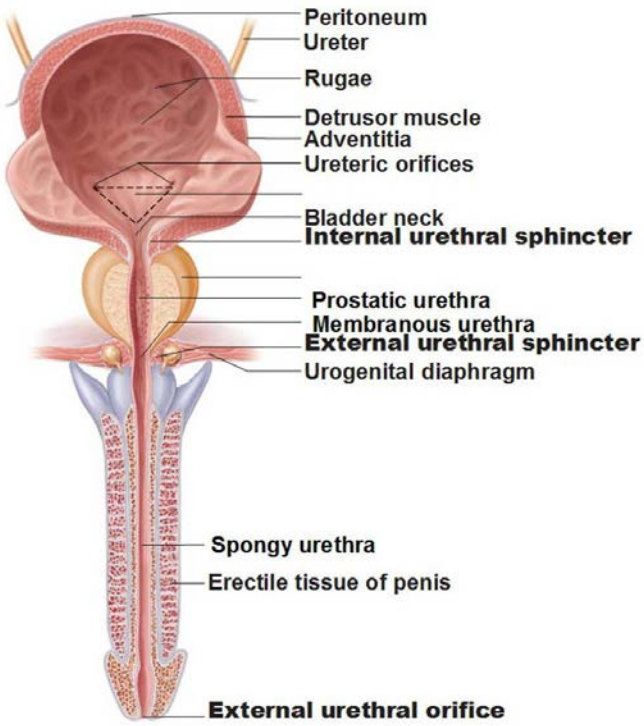


Figure 1.5.1: The male urethra in relation to the urinary bladder.

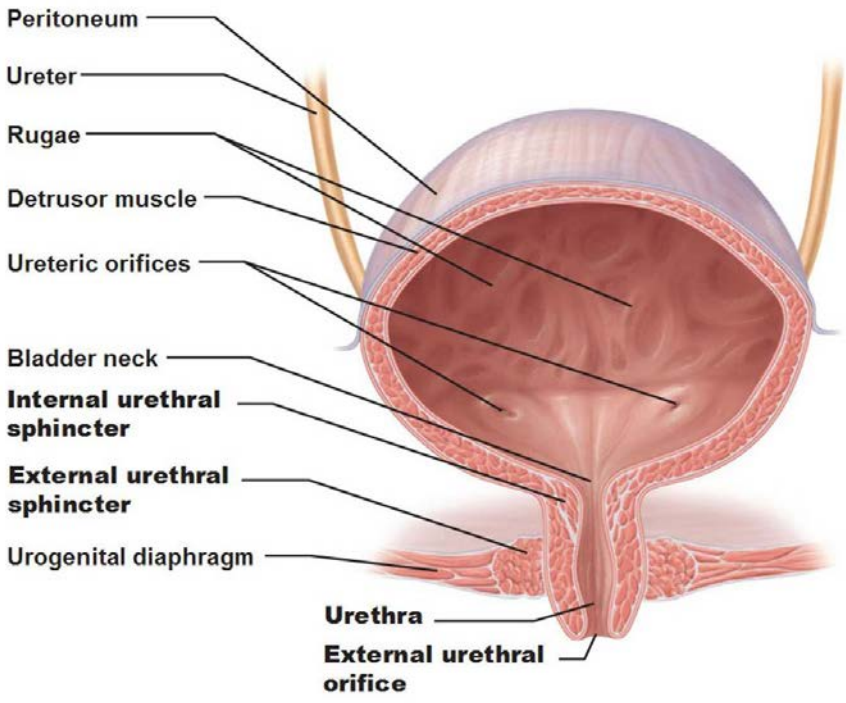


Figure 1.5.2: The female urethra in relation to the urinary bladder.

1.6. Physiology of urination

Urination, also termed micturition, is the process by which the bladder becomes devoid of the urine stored in it. This process includes (i) the progressive filling of the bladder with urine that has been flowing from the renal pelvis through the ureters and (ii) a neuronal reflex called the micturition reflex, which causes emptying of the bladder. The micturition reflex is a spinal cord reflex; it can also be inhibited or promoted by centers in the brainstem and cerebral cortex [2]. Filling of the bladder causes the bladder wall to stretch and contract, as a result of a reflex originating from stretch receptors localized on the bladder wall.

Sensory signals from the bladder fundus subsequently enter the spinal cord via pelvic nerves and return directly to the bladder through parasympathetic fibers in the same nerves; this stimulation of parasympathetic fibers causes intense contraction of the detrusor urinae muscle. Stimulation of the detrusor muscle in turn results in the contraction of the muscle cells in the bladder neck, as the smooth muscle in the bladder is a syncytium. Because the muscle fibers of the bladder outlet are oriented longitudinally and radially, contraction opens the bladder neck and permits urine flow through the posterior urethra. Voluntary relaxation of the external sphincter, achieved by cortical inhibition of the pudendal nerve, allows urine flow through the external meatus. Voluntary relaxation of the external sphincter is required and may be the event triggering micturition. Interruption of the hypogastric sympathetic nerves and the pudendal nerves to the lower urinary tract does not alter the micturition reflex. On the contrary, destruction of the parasympathetic nerves results in complete bladder dysfunction (Figure 1.6.1) [2].

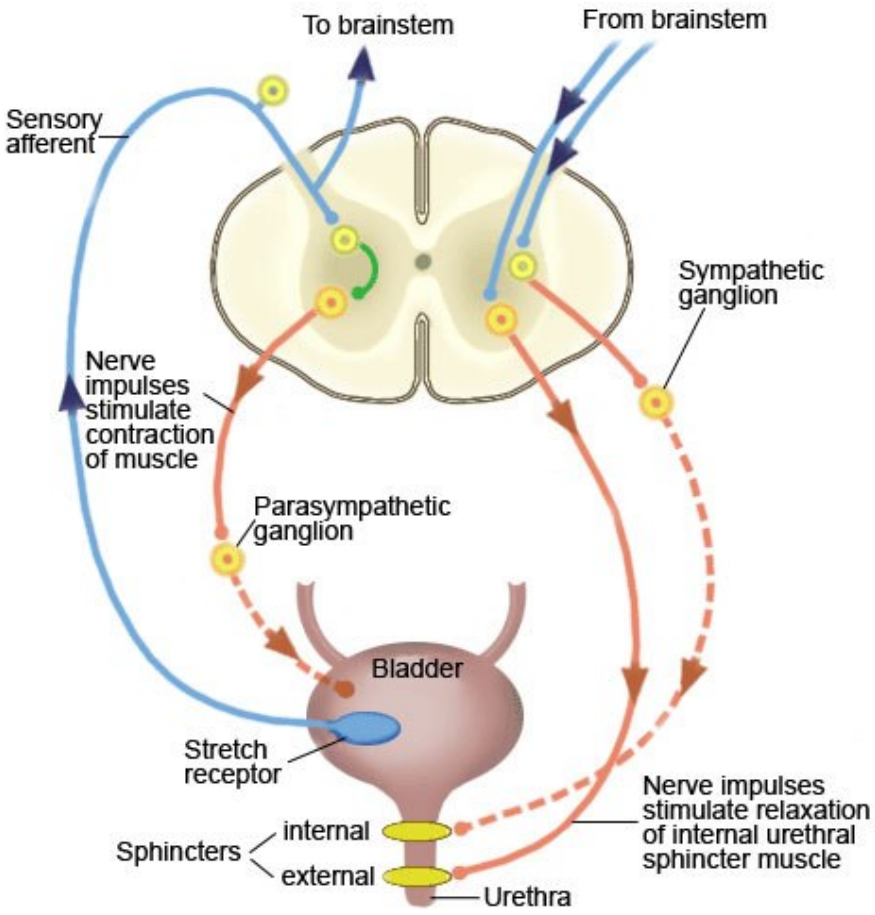


Figure 1.6.1: The neural control of micturition. (Image adapted from McGill Molson Medical Informatics Project)

1.7. Histology of the urothelium

The mucous coat of the urinary bladder is lined with a specific type of epithelial tissue, termed the urothelium. The distinguishing characteristic of urothelium is its ability to sustain fluctuations of volume such as those occurring as the bladder is filled with and emptied of urine. Its alternative designation as transitional epithelium is attributed to its ability to alter its configuration and transit from a multilayered type when the bladder wall is relaxed to a type of ostensibly fewer layers when the bladder wall is distended. Five to six cell layers are present when the bladder is distended, whereas six to eight layers are recognized at the relaxed state. The basal cells rest on the lamina propria, are of small size and are followed by intermediary cells, which are irregular in shape and surrounded by wide intercellular spaces. The superficial cells span the

intermediate cells like an umbrella, which is why they are often termed umbrella cells. They are multinucleated and of flattened shape, although they may also appear as cuboidal cells in the distended bladder (Figure 1.7.1) [5, 6].

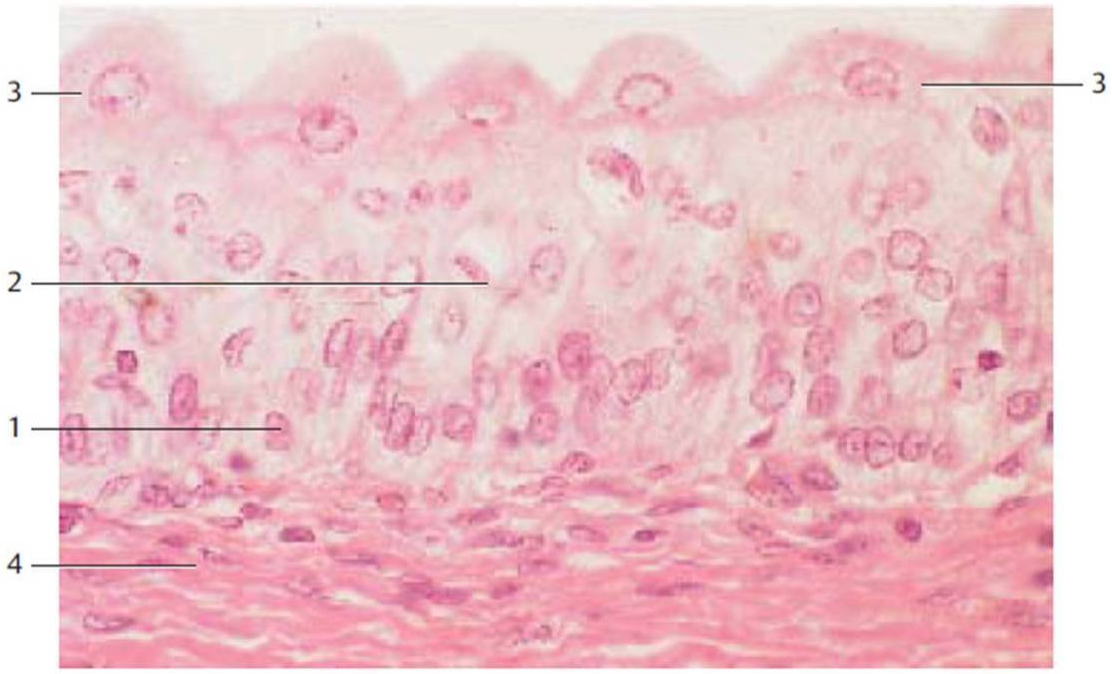


Figure 1.7.1: Microscopic image of the normal urothelium, depicting the basal cells (1), the intermediary cells (2), the superficial cells (3) and the lamina propria (4) [5].

2. URINARY BLADDER CANCER

2.1. Epidemiology of urinary bladder cancer

2.1.1. Incidence

Urinary bladder cancer is the 11th most common malignancy worldwide and the 4th most common among males in the United States, where 74,690 new cases will be diagnosed only in 2014 [7]. Males are affected more frequently than females, with a ratio of 3:1 [8]; moreover, women who have given birth have a lower risk of developing bladder cancer [9], probably due to hormonal changes occurring during pregnancy [10]. On the other hand, large cohort studies have suggested that females are more likely to present with disease recurrence [11]. Bladder cancer tends to affect elderly individuals, with median age at diagnosis being 72 and 74 years for males and females respectively [8]. Studies including thousands of patients have also demonstrated that there is a 2-fold higher incidence of bladder cancer among European Americans than African Americans [12]; yet, individuals of the latter race group present with higher disease stage than European Americans, Hispanics and Asian/Pacific Islanders and five- and ten- year survival is consistently worse for African Americans [12, 13].

2.1.2. Mortality

Urinary bladder cancer is the 9th leading cause of cancer-related death in the United States, leading to 7.7 and 2.2 deaths per 100,000 male and female individuals respectively [14]. On the other hand, females are more likely to succumb to the disease than males [11].

2.2. Environmental risk factors

2.2.1. Tobacco smoking

Tobacco smoking is the primary risk factor for bladder cancer; it has been estimated that ~65% of bladder cancer in males and 20%-30% in females is caused by cigarette smoking [15]. Current smokers of both sexes run a 4-fold higher risk of developing the disease than non-smokers [16] and this risk increases with intensity (number of cigarettes per day) and duration (number of years) of smoking [10]. Other smoking-related characteristics, such as type of tobacco used (black vs. blonde), filtered vs. unfiltered cigarettes and inhalation patterns (deep or not) may modify the risk of bladder cancer [17]. Although the chemicals present in tobacco smoke that are associated with the disease have yet to be determined, studies have recognized amines such as 2-naphthylamine, 4-aminobiphenyl and benzidine as potential bladder carcinogens [18]. Smoking cessation can decrease the risk of bladder cancer by over 30% in 1-4 years [19], further highlighting the causative link between tobacco use and bladder cancer, as well as the physicians' role in counseling patients on smoking cessation. However, even after 25 years of smoking cessation, the risk of the ex-smokers remains higher than this of the never-smokers [19].

Metabolism of carcinogens contained in tobacco products can modify the risk of smoking-related bladder cancer. N-acetyltransferases 1 and 2 (*NAT1* and *NAT2*) are enzymes involved in the detoxification of aromatic and heterocyclic amines, by catalyzing their acetylation. It has been demonstrated that smokers with the slow *NAT2* acetylation phenotype have an increased risk of bladder cancer; rapid *NAT1* acetylation phenotype may also contribute to the disease in smokers who are already slow *NAT2* acetylators [20].

2.2.2. Occupational exposure

Occupational exposure, the first recognized risk factor for bladder cancer and the second most important, is responsible for almost 20% of all bladder cancer cases [10].

Historically, the first bladder cancer cases in aniline dye factory workers were reported in 1895 [21] and the first study investigating the risk linked to aromatic amines was published in 1954 [22]. Since then, exposure to aromatic amines such as 2-naphthylamine, 4-aminobiphenyl, 4-4-diaminobiphenyl (benzidine), 4,4´-methylenedianiline, 4,4´-methylene-bis(2-chloroaniline), o-toluidine, 4,4´-methylene bis(2-methylaniline), and 4-chloro-o-toluidine has been associated with bladder carcinogenesis [10, 23].

Workers in the textile dye, painting and leather industries, as well as autoworkers, truck drivers, metalworkers, paper and rubber manufacturers, dry cleaners, dental technicians, hairdressers, and marine engineers have therefore been marked as potentially high-risk groups [8]. In most cases, long periods of 30 to 50 years after exposure are required for the aforementioned chemicals to exert their carcinogenic effect on the bladder [24]. Many of them have been banned from work environments to minimize risk from occupational exposure, yet others (such as orthotoluidine) continue to be used in chemical industries and to cause disease [25].

It has also been suggested that exposure to polycyclic aromatic hydrocarbons (PAHs) can contribute to bladder cancer; a study reported that approximately 4% of bladder cancer cases in Western European men are due to exposure to PAHs [26]. A moderate increase in the risk of bladder cancer due to exposure to PAHs has also been observed among workers in coal gasification, aluminum production and iron and steel foundries [27]. PAHs are also present in diesel exhaust, exposure to which has previously been shown to moderately increase the risk of bladder cancer [28].

A recent meta-analysis has also shown an increased risk of bladder cancer in hairdressers or related workers in the cosmetic industry, due to exposure in potential carcinogens contained in hair dyes, sprays and other hair care products [29].

Indicative of the contribution of occupational exposure to incidence of the disease is the fact that bladder cancer has long been characterized as a prescribed industrial disease in the United Kingdom [30].

2.2.3. Schistosomiasis

Schistosomiasis, also known as bilharzia, is an infection caused by parasitic worms of the *Schistosoma* genus. Squamous cell carcinoma (SCC) of the bladder has been reported as the leading maligancy in certain geographical regions in which schistosomiasis is common, predominantly in countries of the Middle East and the sub-Saharan Africa. The species *Schistosoma haematobium* is detected more frequently in patients with SCC of the bladder, whereas in Egypt mixed infection with *Schistosoma haematobium* and *Schistosoma mansoni* is endemic [31]. The parasite inhabits the veins around the bladder and its eggs can infiltrate the bladder wall; some of them are not excreted in urine and remain in the tissue, where they initiate a chronic inflammatory reaction, along with physical damage which triggers increased cell turnover and urothelial hyperplasia [32]. Also, increased concentrations of nitrosamines, which are known carcinogenic compounds, have been measured in patients infected with *Schistosoma haematobium*, probably due to macrophage accumulation [33]. In addition, a *Schistosoma*-infected bladder is more susceptible to genomic instability, hence to malignant transformation, due to increased frequency of *p53* inactivation [34].

2.2.4. Chronic urinary tract infection

Squamous cell carcinomas of the bladder have been associated with chronic urinary tract infections (UTIs), which in turn may be triggered by bacteria, catheters or bladder calculi [35]. Patients with spinal cord injury, who have an indwelling catheter for more than 10 years, run a higher risk of developing the disease, which will originate from squamous metaplasia of the bladder in 80% of the cases [36]. In these cases, increased cell proliferation is thought to be promoted by chronic inflammation and mechanical stimuli from the catheter [10, 36]. Chronic irritation and/or inflammation of the bladder can also result from bladder calculi and it has recently been reported that bladder cancer patients (especially males) are more likely to have been diagnosed with bladder calculi when compared to bladder cancer free individuals [37].

Viral infections have at times been implicated in the etiology of bladder cancer. A large cohort study reported association of genital warts caused by HPV infection with an increased risk of bladder cancer [38], whereas Epstein-Barr virus and members of the Polyoma virus family have been detected in bladder tumors [39].

2.2.5. Cyclophosphamide

Cyclophosphamide is an alkylating chemotherapeutic agent used in the treatment of lympho- and myeloproliferative diseases, as well as some autoimmune disorders and has long been associated with the development of transitional cell carcinoma of the bladder in non-Hodgkin's lymphoma patients [40]. Among the substances produced following the metabolism of cyclophosphamide, acrolein is highly toxic and is suspected to have a carcinogenic effect on the bladder. Particularly when administered orally, cyclophosphamide can cause hemorrhagic cystitis in patients with rheumatic diseases, who also run an increased risk of bladder cancer years later. To prevent hemorrhagic cystitis, a thiol compound called mesna is prescribed, although its effectiveness in preventing bladder cancer has been questioned [41].

2.2.6. Radiotherapy

Radiotherapy in adjacent organs is also a known risk factor for bladder cancer. An increased risk of bladder cancer was observed among testicular cancer survivors treated with radiation [42]. Also, prostate cancer patients who received radiotherapy were found to run a higher risk of muscle-invasive urothelial carcinoma than those treated with other methods [43]. Similarly, a high risk of bladder malignancies was observed in women treated with radiotherapy for cervical cancer 40 or more years before [44].

2.2.7. Diet

A diet rich in fruit has been associated with a small but significant decrease in bladder cancer risk [45]; on the contrary, vegetables have not been found to have a protective effect. In addition, an increased fluid intake has been associated with reduced bladder cancer risk in men, as it increases the frequency of micturition and thus decreases the exposure of the urothelium to potentially carcinogenic substances [46].

The relation of coffee and tea consumption with the risk of bladder cancer has often been investigated, with inconsistent results. Most of these inconsistencies are attributed to confounding by smoking status, as coffee consumption and cigarette smoking are strongly correlated. In a large prospective study on Japanese individuals, coffee consumption was positively associated with bladder cancer risk in men who never smoked or had quit and daily green tea consumption of 5 cups or more was found to increase the risk of the disease in women, regardless of smoking status [47]. However, meta-analyses of cohort and case-control studies have suggested that there is either no significant association between coffee consumption and bladder cancer risk or that evidence supporting this association is inconclusive [48, 49]. On the other hand, a recent study employing mouse models demonstrated that polyphenols contained in green tea do not have anti-tumor effects in the bladder, yet may act as inhibitors of tumor growth and invasion [50]. Although coffee or tea consumption are not established risk factors for bladder cancer, further research would elucidate their role in this type of malignancy.

Artificial sweetener consumption has also been suspected of enhancing the risk of bladder cancer, especially after it was shown that saccharin can increase bladder cancer risk in rats [51]. A later case-control study found that high sweetener consumption correlated with a modest increase in bladder cancer risk and with a more aggressive tumor phenotype [52]. Nevertherless, there has not yet been conclusive scientific evidence designating artificial sweeteners as bladder carcinogens.

Exposure to arsenic has also been implicated in bladder carcinogenesis, since incidence of transitional cell carcinomas was found to be significantly higher, in a dose-dependent fashion, among residents of an area in northwestern Taiwan, where high levels of the substance in the water are endemic [53]. According to a recent meta-

analysis, exposure to low levels of arsenic in drinking water is not likely to affect bladder cancer risk, particularly among never smokers [54].

2.2.8. Analgesic and anti-inflammatory drug use

A recent meta-analysis has showed no association between acetaminophen or aspirin use and bladder cancer risk, although use of non-aspirin anti-inflammatory drugs could probably have a protective effect in nonsmokers [55]. A previous case-control study reported a significantly decreased bladder cancer risk in regular anti-inflammatory drug users, but an increased risk among phenacetin users, especially in long-term ones [56]. Phenacetin has long been classified as probably carcinogenic to humans and its association with bladder cancer incidence has led to its withdrawal from the market in countries like the United States and Japan [56, 57].

2.2.9. Family history

The majority of bladder cancer cases are sporadic and familial bladder cancer is rather infrequent. In addition, environmental factors, such as cigarette smoking or exposure to chemicals, may modify familial risk. In a large study on familial bladder cancer in the Netherlands, a familial subtype of the disease was recognized, with an almost 2-fold increased risk among first-degree relatives [58]. Another case-control study in Italy reported an up to 6-fold increased risk of bladder cancer in individuals with affected first-degree relatives [59].

A list of environmental risk factors of bladder cancer is shown in Table 2.2.1.

Table 2.2.1. Alphabetical list* of agents, mixtures or exposure circumstances associated with bladder cancer [73].

Aluminum production
4-Aminobiphenyl
Analgesic mixtures containing phenacetin
Arsenic in drinking water
Auramine manufacture
Benzidine
Chlornaphazine
Coal gasification
Coal-tar pitch
Cyclophosphamide
Magenta manufacture
2-Napthylamine
Rubber industry
Schistosoma haematobium (infection)
Tobacco smoke

*Compiled from the IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. The above exposures have been classified into IARC Group-1 (carcinogenic to humans).

2.3. Diagnosis

2.3.1. Signs and symptoms

Painless hematuria, macroscopic or microscopic, occurs in up to 85% of patients and is the most common presenting symptom of bladder cancer [10, 60]. Hematuria, however, may as well occur in benign conditions of the urinary tract, such as infections, other inflammatory conditions, nephrolithiasis or benign prostatic hyperplasia [8]. Hematuria may also be misinterpreted as a symptom of urinary tract infections in females, which can result in delayed diagnosis of bladder cancer [61]. Patients may also present with irritative voiding syndromes, such as frequency, dysuria and urgency or with flank pain associated with ureteral obstruction, lower extremity edema and palpable pelvic mass. Symptoms such as weight loss and abdominal or bone pain indicate advanced disease and very rarely occur without hematuria [10].

2.3.2. Cystoscopy

Cystoscopy is the endoscopic examination of the bladder and is considered as the "gold standard" for the detection of bladder cancer. This examination provides the urologist with useful information which is implemented on patient management, such as location and number of tumors, as well as size and shape (Figure 2.3.1) [10]. Nevertheless, flat lesions such as carcinoma *in situ* might be overlooked during conventional cystoscopy, which may result in incomplete surgical resection and higher recurrence rates. Use of fluorescent agents like 5- aminolevulinic acid (5-ALA) has been shown to improve the effectiveness of the initial resection, at least in non-muscle-invasive bladder cancer. In a prospective 8-year study, patients screened with 5-ALA-induced fluorescence cystoscopy exhibited lower residual tumor rate and higher recurrence-free survival than those screened with the conventional examination [62]. Virtual cystoscopy is a non-invasive technique which allows visualization of precise spatial relations between cancerous and normal adjacent tissue, yet is costly and rather ineffective in depicting flat lesions [63].

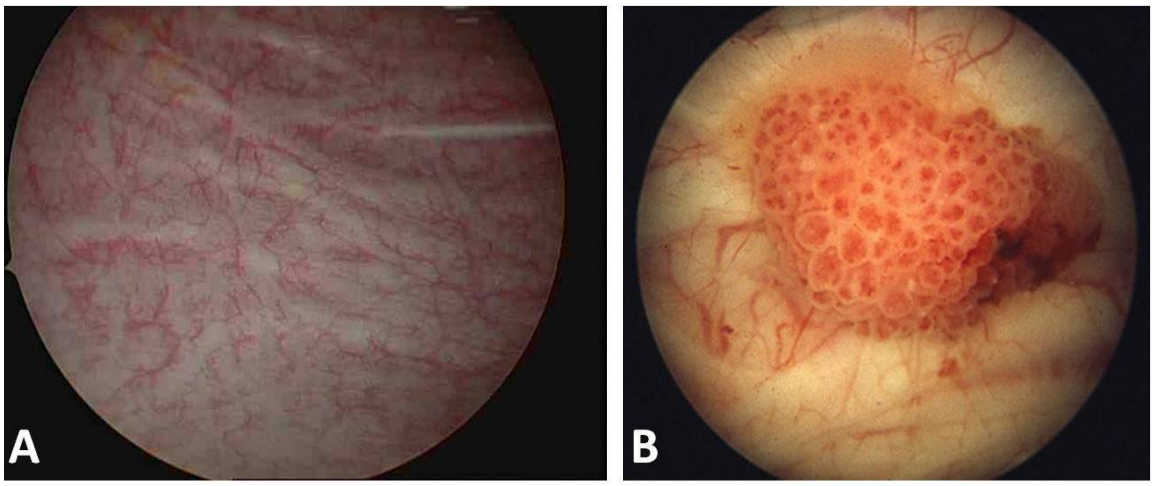


Figure 2.3.1: Cystoscopy images of **(A)** normal bladder urothelium and **(B)** non-muscle-invasive bladder tumor.

2.3.3. Imaging

Radiographic imaging of urinary bladder tumors is essential for pre-treatment staging and is performed using computed tomography (CT) or magnetic resonance (MR) techniques [8]. Gadolinium-enhanced MRI is highly accurate in distinguishing non-muscle-invasive from muscle-invasive lesions, as well as organ-confined from non-organ-confined disease [64]. Continued advances in CT and MRI may help to improve staging and to meet the challenge of accurate detection of early metastatic disease [63].

2.4. Staging

2.4.1. Clinical staging

Clinical staging is performed pre-operatively and involves the estimation of the extent of local spread of the tumor in the bladder wall. It is based on cytoscopic examination and bimanual examination under anaesthesia. Clinical staging is highly important for the evaluation of bladder cancer patients and it has been estimated that up to 50% of patients may be clinically understaged [65].

During the initial endoscopic examination, the bladder, urethra and the upper urinary tract are evaluated for potential disease. Bladder biopsy, conventional or cold-cup, or transurethral resection of bladder tumor (TURBT) is subsequently performed for the removal of tissue, which is sent for pathologic examination. In order to prevent understaging and disease progression, a second TURBT is recommended for patients with high-grade urothelial carcinoma of stages pTa-pT1 [66]. As already mentioned, imaging essentially contributes to clinical staging.

2.4.2. Pathological staging

Pathological staging is carried out by the histological examination, with the use of microscope, of the tissue that has been removed on biopsy or resection and involves the determination of the precise depth of extension of the tumor into the bladder wall. The TNM (Tumour, Node, Metastasis) classification of malignant tumors is widely applied to classify the extent of cancer and is also used in the pathological staging of urinary bladder tumors (Table 2.4.1) (Figure 2.4.1) [67].

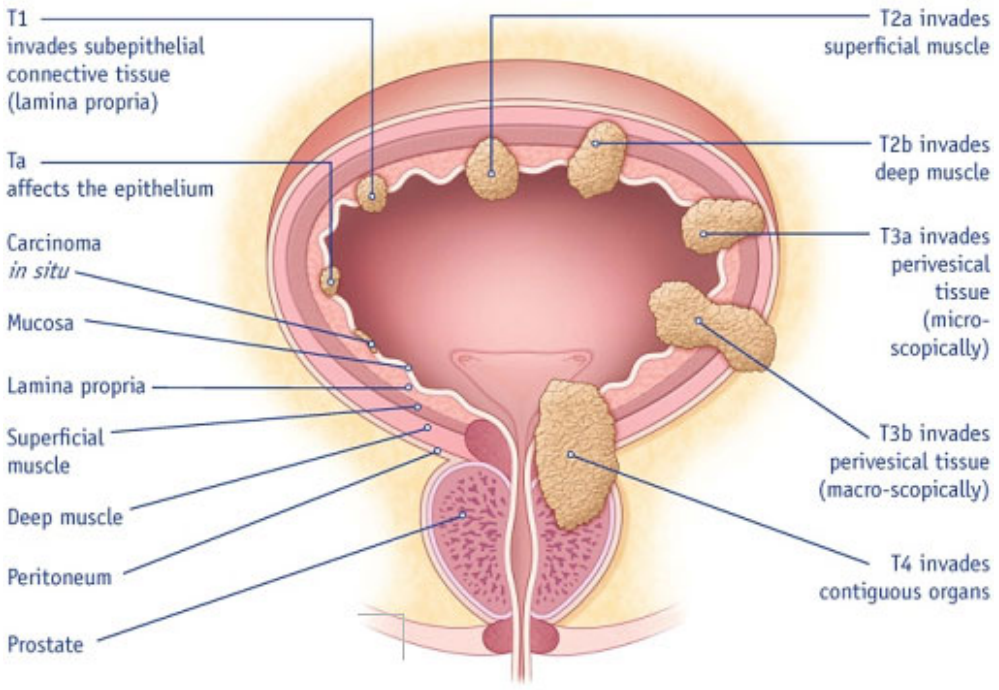


Figure 2.4.1: Urinary bladder cancer staging.

Table 2.4.1: 2009 TNM classification of urinary bladder cancer

T - *Primary tumor*

TX Primary tumor cannot be assessed

T0 No evidence of primary tumor

Ta Non-invasive papillary carcinoma

Tis Carcinoma *in situ*: 'flat tumor'

T1 Tumor invades subepithelial connective tissue

T2 Tumor invades muscle

- **T2a** Tumor invades superficial muscle (inner half)
- **T2b** Tumor invades deep muscle (outer half)

T3 Tumor invades perivesical tissue

- **T3a** Microscopically
- **T3b** Macroscopically (extravesical mass)

T4 Tumor invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall

- **T4a** Tumor invades prostate, uterus or vagina
- **T4b** Tumor invades pelvic wall or abdominal wall

N – *Lymph Nodes*

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac or presacral)

N2 Metastasis in multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac or presacral)

N3 Metastasis in a common iliac lymph node(s)

M – *Distant Metastasis*

MX Distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis

2.5. Histopathology

2.5.1. Histological types of bladder tumors

It has been estimated that 90-95% of bladder cancer cases are urothelial carcinomas (Figure 2.5.1.A, Figure 2.5.1.B), whereas the remaining 5-10% of non-urothelial tumors comprise of squamous cell carcinomas, adenocarcinomas or malignant lesions originating from cell types other than epithelial (small cell carcinomas, sarcomas etc) [68, 69].

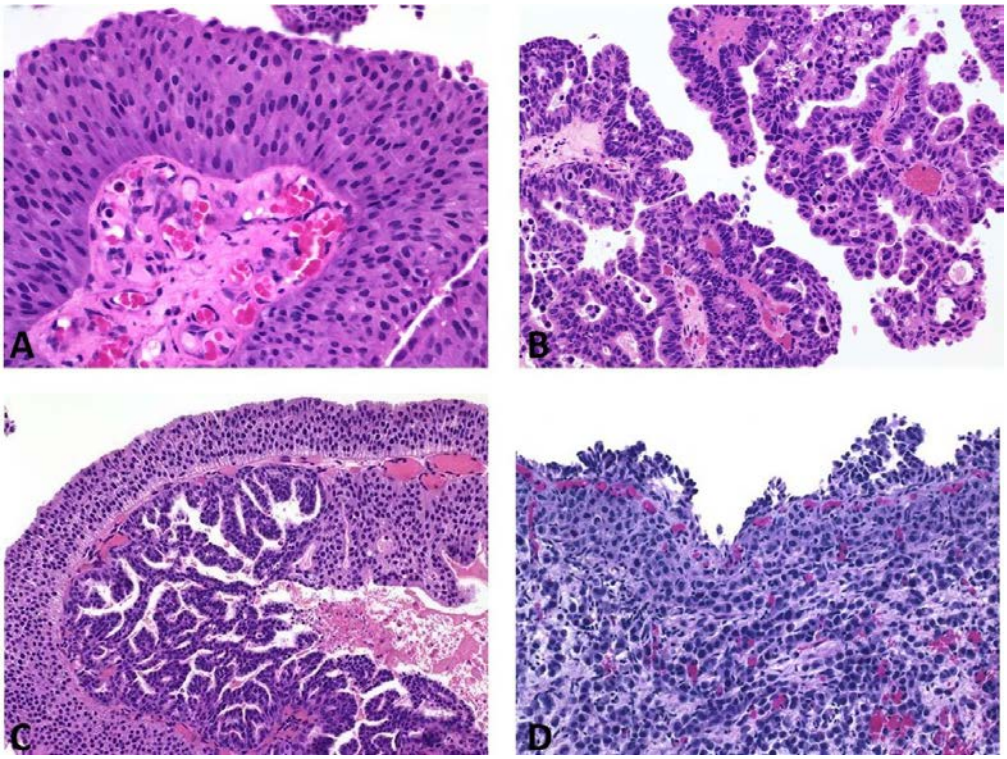


Figure 2.5.1: (A) Low-grade transitional cell carcinoma of the bladder with scattered hyperchromatic nuclei and typical mitotic figures. **(B)** High-grade transitional cell carcinoma of the bladder with enlarged nuclei which show variably increased chromatin content. **(C)** Micropapillary variant of urothelial carcinoma; the transition from "conventional" papillary urothelial carcinoma to the micropapillary pattern is notable. **(D)** Plasmacytoid variant of urothelial carcinoma, in which the tumor cells closely resemble plasma cells; in this example, the overlying papillary component is partially denuded. (WHO/ISUP Classification, 2004) (© WebPathology.com)

Although urothelial tumors are mostly encountered in their pure form, they may also present with mixed histologic features. Histologic variants of urothelial tumors have been reported with squamous, glandular, micropapillary and plasmacytoid differentiation (Figure 2.5.1.C, Figure 2.5.1.D). It has been suggested that urothelial tumors with divergent differentiation are more likely to be invasive than pure urothelial ones [69].

Squamous cell carcinomas account for approximately 3% of bladder cancer cases and are strongly associated with *Schistosoma* infection [31]. They often present at an advanced stage and are thought to be more aggressive than urothelial carcinomas (Figure 2.5.2) [70].

Adenocarcinomas account for 0.5-2% of all bladder tumors, are associated with bladder exstrophy and *Schistosoma* infection and mostly present as solitary lesions. Histologic subtypes such as signet ring cell and clear cell adenocarcinomas of the bladder are more aggressive (Figure 2.5.3A). Urachal adenocarcinomas are extremely rare tumors that arise from remnants of the urachus in the bladder (Figure 2.5.3B) [71].

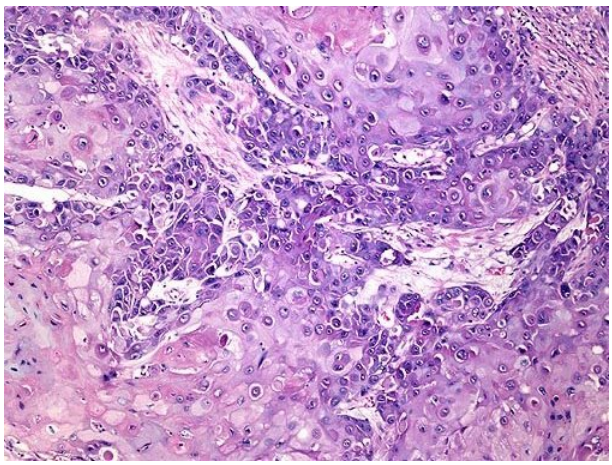


Figure 2.5.2: Moderately differentiated squamous cell carcinoma (WHO/ISUP Classification, 2004) (© WebPathology.com)

Small cell carcinomas represent 1% of the cases undergoing cystectomy and, like squamous cell carcinomas, present at an advanced stage [69].

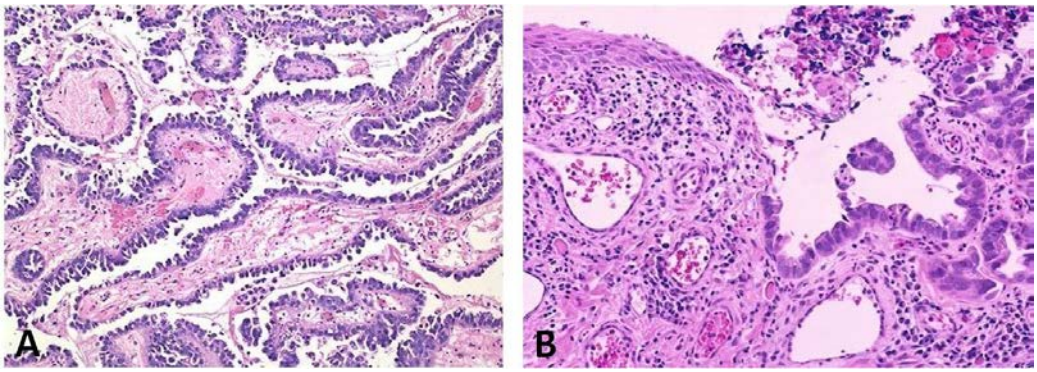


Figure 2.5.3: (A) Clear cell adenocarcinoma of the bladder; in this example, the tumor cells are arranged in papillary structures. **(B)** Urachal adenocarcinoma with papillary and glandular differentiation; this bladder tumor extended along the urachal tract to produce ulceration around umbilicus (umbilical skin is seen at upper left). (© WebPathology.com)

Carcinoma *in situ* (CIS) of the urinary bladder is a high-grade non-muscle invasive pre-cancerous lesion (Figure 2.5.4), with 20-83% of the cases progressing to muscle-invasive carcinoma. Frequently multifocal, it is mainly of urothelial origin and rarely presents as an *in situ* adenocarcinoma, although the latter has been described as more aggressive. Urothelial CIS is often designated as "secondary CIS", as it is encountered in conjunction with muscle-invasive urothelial tumors in 45-65% of the cases. Primary CIS account for 1-3% of all urothelial tumors [72].

2.5.2. Histological grading of urothelial neoplasms

Pathologists use histological grading in order to evaluate the degree of anaplasia of tumor cells. The 1973 World Health Organization (WHO) classification system has been the most widely used for grading of urothelial tumors [73]. A new classification system was initially proposed by the WHO/ISUP in 1998 and updated by the WHO in 2004 (Table 2.5.1) [74].

The majority of studies and clinical trials carried out so far have used the 1973 WHO Classification. Until the 2004 WHO classification has been validated by more clinical trials, tumors should be graded using both the 1973 and the 2004 WHO Classifications. Grade 1 (well-differentiated) tumors were described by Mostofi et al as having the least degree of cellular anaplasia compatible with a diagnosis of malignancy, in contrast with grade 3 (poorly differentiated) tumors which have the most severe degrees of anaplasia; grade 2 (moderately differentiated) tumors lie in between [73].

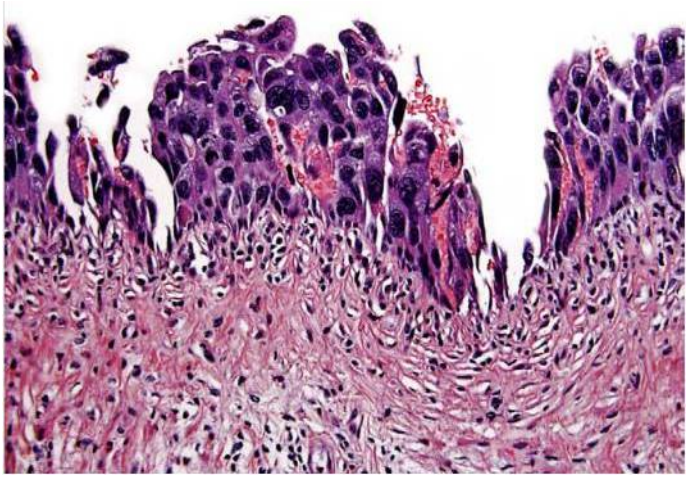


Figure 2.5.4: Urothelial carcinoma *in situ* (CIS) with markedly enlarged hyperchromatic urothelial cell nuclei and a normal to slightly increased number of cell layers [72].

Papillomas are typically solitary, exophytic tumors, composed of a delicate fibrovascular core covered by normal-looking urothelium (Figure 2.5.5.A); their incidence and recurrence rates are low [74]. Papillary urothelial neoplasms of low malignant potential (PUNLMPs) exhibit increased cellular proliferation exceeding the thickness of normal urothelium, yet they lack cytologic features of malignancy (Figure 2.5.5.B) [75]. The low-grade papillary urothelial carcinoma group includes all former grade 1 (WHO 1973) cases and some former grade 2 cases.

Table 2.5.1: 1973 and 2004 WHO grading of urothelial neoplasms.

1973 WHO grading
▪ Urothelial papilloma
▪ Grade 1: well-differentiated tumors
▪ Grade 2: moderately differentiated tumors
▪ Grade 3: poorly differentiated tumors
2004 WHO grading
▪ Urothelial papilloma
▪ Papillary urothelial neoplasm of low malignant potential (PUNLMP)
▪ Low-grade papillary urothelial carcinoma
▪ High-grade papillary urothelial carcinoma

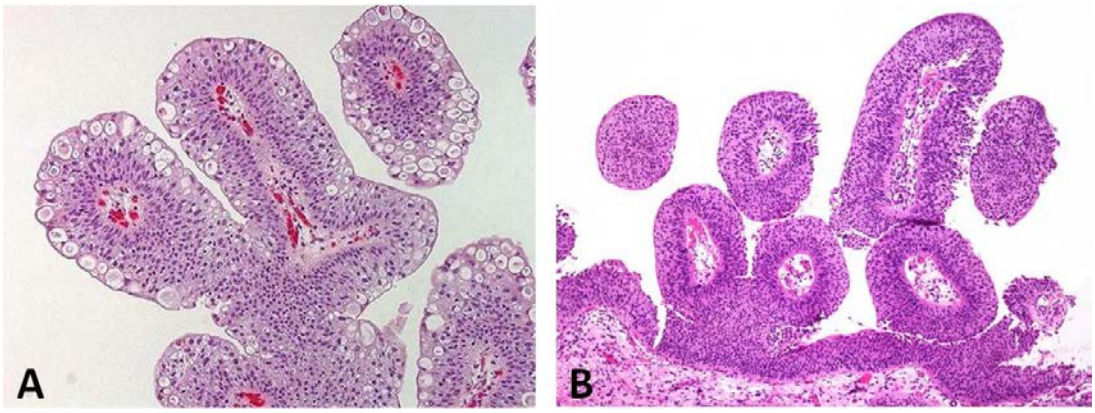


Figure 2.5.5: The major distinction between PUNLMP and urothelial papilloma is thickening of the urothelium in the former. **(A)** Urothelial papilloma with papillary fronds containing delicate fibrovascular cores lined by less than 7 layers of cytologically and architecturally normal urothelium; prominent cytoplasmic vacuoles are also seen. **(B)** PUNLMP, characterized by a homogenous monotonous appearance, normal to slightly enlarged nuclei and inconspicuous nucleoli. (WHO/ISUP Classification, 2004) (© WebPathology.com)

2.6. Treatment and surveillance

Bladder cancer patients are treated with surgery, intravesical chemo- or immunotherapy and radiotherapy. Surgery is implemented in nearly all cases and a combination of these treatment types is frequently applied.

Low-grade bladder papillomas are treated with transurethral resection of bladder tumor (TURBT), which is a method of diagnostic, prognostic and often therapeutic value [76]. According to NCCN guidelines, administration of a single dose of intravesical chemotherapy within 24 hours of resection is recommended. Bacillus Calmette-Guérin (BCG), an anti-tuberculosis vaccine, has also been shown to be effective in the prevention of recurrences. Although the risk for progression is low, these patients are followed-up with cystoscopy at 3-month intervals [77].

Transurethral resection of bladder tumor is the standard treatment for non-muscle-invasive pTa and pT1 carcinomas and CIS [77]. After an initial TURBT, some of the patients may still have residual disease, which increases their risk for early recurrence and progression. Thus, the identification of residual disease is highly important and calls for the performance of a repeat TURBT. Patients diagnosed with high-grade pTa or any pT1 tumors, as well as patients with incompletely resected lesions, undergo a repeat TURBT 2 to 6 weeks after the initial one [66, 76]. Depending on the stage and grade of the lesion, intravesical therapy may be recommended to prevent recurrence or progression to a higher stage or grade. Intravesical therapy is used either as adjuvant therapy after a complete resection or as a means of eradicating residual tumors [77]. It has been shown, however, that intravesical chemotherapy may not compensate for incomplete resection in pT1 tumors [78].

For high-grade pTa and pTis lesions, the NCCN Bladder Cancer Panel recommends BCG as the preferred option for adjuvant treatment and a close follow-up with urinary cytology and cystoscopy at 3-month intervals, along with imaging of the upper urinary tract [76]. Incomplete resection of high-grade or multifocal pT1 lesions is addressed with repeat TURBT, intravesical therapy with BCG or mitomycin C (MMC) or with early cystectomy, which is also performed if the tumors do not respond to immuno- or chemotherapy. In case of persisting or recurrent pTa and pTis lesions after a second

course of treatment with BCG or MMC, a cystectomy or use of a different intravesical agent (e.g. MMC, valrubicin or BCG plus interferon-α) is recommended [77].

Patients with muscle-invasive pT2a and pT2b disease are treated with radical cystectomy and chemotherapy. The surgical procedure involves cystoprostatectomy in males and cystectomy and hysterectomy in females, resection of pelvic lymph nodes, as well as the formation of urinary diversion. Partial cystectomy is performed in fewer than 5% of the cases and is recommended for tumors which have developed on the dome of the bladder and have no associated Tis in other areas of the urothelium. Follow-up after a cystectomy includes urine cytology, chest radiograph, imaging of the abdomen and pelvis, CT scan, liver function and other biochemical tests. Radiotherapy is not used alone but in combination with neoadjuvant and concurrent chemotherapy. Agents such as neoadjuvant MCV (methotrexate, cisplatin, and vinblastine) and 5-fluorouracil (5-FU) have produced promising results when combined with radiotherapy in the treatment of pT2 lesions after TURBT. As for neoadjuvant chemotherapy before cystectomy of organ-confined bladder tumors, the NCCN Bladder Cancer Panel recommends cisplatin-based combinations, such as methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC), cyclophosphamide, doxorubicin, and cisplatin (CAP) and methotrexate, vinblastine, epirubicin, and cisplatin (MVEC) [77].

Non-organ confined disease (stages T3a, T3b/T4a, T4b) calls for radical cystectomy with consideration of cisplatin-based combination neoadjuvant chemotherapy. Especially for tumors with nodal involvement or vascular invasion, which are likely to relapse, adjuvant chemotherapy or radiotherapy are recommended. Follow-up is carried out according to the guidelines mentioned previously for high-grade lesions. Patients with unresectable pT4 disease are considered for chemotherapy alone or radiotherapy in combination with chemotherapy, usually 5-FU- or cisplatin-based [77].

Systemic chemotherapy and/or radiotherapy are recommended for patients who present with or subsequently develop metastatic disease, after evaluation by chest CT, bone scan, and determination of creatinine clearance. Cisplatin, the taxanes and gemcitabine or combinations of these agents are used with clinical benefit, depending on the presence or absence of medical comorbidities (cardiac disease, renal dysfunction), along with the risk classification of the patient based on disease extent. Patients with metastatic disease are re-evaluated after 2 to 3 cycles of chemotherapy,

and treatment is continued for 2 more cycles in patients whose disease responds or remains stable [77].

Alhough clinical experience with chemotherapy in non-urothelial carcinomas is limited, it has been observed that the regimens applied in the treatment of urothelial carcinoma are not effective. Patients with non-urothelial carcinomas are treated based in the identified histology of the tumor (e.g. adenocarcinomas, squamous cell carcinomas etc) [77].

Bladder sparing, also termed bladder preservation, represents a regime for selected patients with pT2 or pT3a urothelial lesions. It can be carried out by means of aggressive endoscopic TURBT alone, TURBT followed by chemotherapy alone, partial cystectomy, radiotherapy alone, or a combination of chemotherapy and radiotherapy [8, 77]. Patients treated with the last option undergo a cystoscopy with bladder biopsy midway through treatment. If disease is detected, cystectomy is recommended. For all other modalities, repeat TUR is performed 2 to 3 months after induction therapy. If persistent disease is detected, palliative cystectomy is recommended when possible [77]. Bladder-preserving regimes are reasonable alternatives to cystectomy for patients who are medically unfit for surgery and for those seeking an alternative treatment plan. Nevertheless, these strategies depend on TURBTs before and after treatment and call for lifelong surveillance. Other disadvantages include the risk of radiation and chemotherapy toxicities and the need for salvage cystectomy in case of treatment failure [8, 77].

2.7. Prognosis

It is generally accepted that the most important factors that impact the prognosis of bladder cancer are the grade and stage of the tumor. Tumors of low grade and/or stage are associated with more favorable prognosis and survival. Other factors of the patient risk profile influencing prognosis are number and size of tumors, recurrence, response to initial treatment and conjunct CIS.

Urothelial papillomas, which are rarely encountered, tend to recur also very rarely (~8%) after resection [74].

Papillary urothelial neoplasms of low malignant potential also come with excellent prognosis, as they recur locally and at significantly lower rates (up to 35%) than non-muscle-invasive papillary carcinomas. In addition, they rarely progress to tumors of higher stage and/or grade [75, 79].

Low-grade, non-muscle-invasive papillary carcinomas present as single tumors in 78% of the cases. They frequently recur, with reported rates of 48-71%, yet rarely progress to invasive, life-threatening lesions (only 5% of the cases) [75].

High-grade, non-muscle-invasive papillary carcinomas exhibit high recurrence rates of up to 78% within 5 years of diagnosis, depending on the patient risk profile. They also have a high risk of progression to muscle-invasive disease (45%), which raises questions about the efficacy of the conservative treatment regime applied in most cases (TURBT and BCG instillations) [80].

Primary CIS is encountered in conjunction with muscle-invasive urothelial carcinomas and with papillary neoplasms. When compared to secondary CIS, it is less likely to progress to muscle-invasive disease. High mortality rates (45-65%) are observed among patients with CIS and conjunct invasive disease, whereas 7-15% of patients with CIS and non-muscle-invasive tumors die because of the disease [81].

The majority of the pT1 urothelial carinomas are papillary and of low or high grade, while most of the ≥ pT2 cases are non-papillary and of high grade. As expected, depth of invasion is the most important prognostic factor and tumors infiltrating beyond the muscularis mucosae have higher progression rates. The risk for recurrence and progression of muscle-invasive lesions increases with tumor multifocality, tumor size of > 3 cm and with concurrent CIS. Other factors associated with an unfavorable outcome are infiltration of the ureteral orifice, lymph node metastases and presence of systemic dissemination [82].

As already mentioned, squamous cell carcinomas and adenocarcinomas of the bladder, as well as tumors of other cell origin, usually present at advanced stages and are associated with poor prognosis.

2.8. Molecular background

It has been established that tumorigenesis in humans is a multistep process involving genomic, genetic and epigenetic aberrations. After years of intense investigation, tumors are currently considered as heterogeneous tissues comprised of distinct cell types constantly interacting with one another rather than isolated cell masses with merely increased proliferative potential [83].

Urothelium, also termed transitional epithelium, is a highly specialized type of epithelial tissue. Its physiological role is to protect underlying structures from toxic substances contained in urine and to accommodate changes in its surface area during the micturition cycle. To be able to maintain the stability required for this dual role, urothelium is one of the slowest cycling epithelia of the human body, with a turnover rate of approximately 200 days [84].

Although tumorigenesis in most epithelial tissues follows a single pathway of mutations in genes that regulate growth, survival, apoptosis and cell-cell interactions [85], this does not apply for bladder epithelium. Malignant transformation of urinary bladder cells follows two distinct molecular pathways and gives rise to two major phenotypic variants of tumors with entirely different biological behaviour. Low-grade non-muscle-invasive tumors, comprising approximately 80% of the cases, have limited invasive potential and come with favorable prognosis when treated early, although accompanied by high recurrence rates. On the contrary, high-grade muscle-invasive tumors have very bad prognosis, with most of the patients developing life-threatening metastases despite surgical resection and systemic therapy [86].

The genetic alterations underlying the development of urinary bladder tumors of both phenotypic variants include inactivation of tumor-suppressor genes, activation of oncogenes, chromosomal aberrations and alterations in the tumor micorenvironment (Figure 2.8.1) [86].

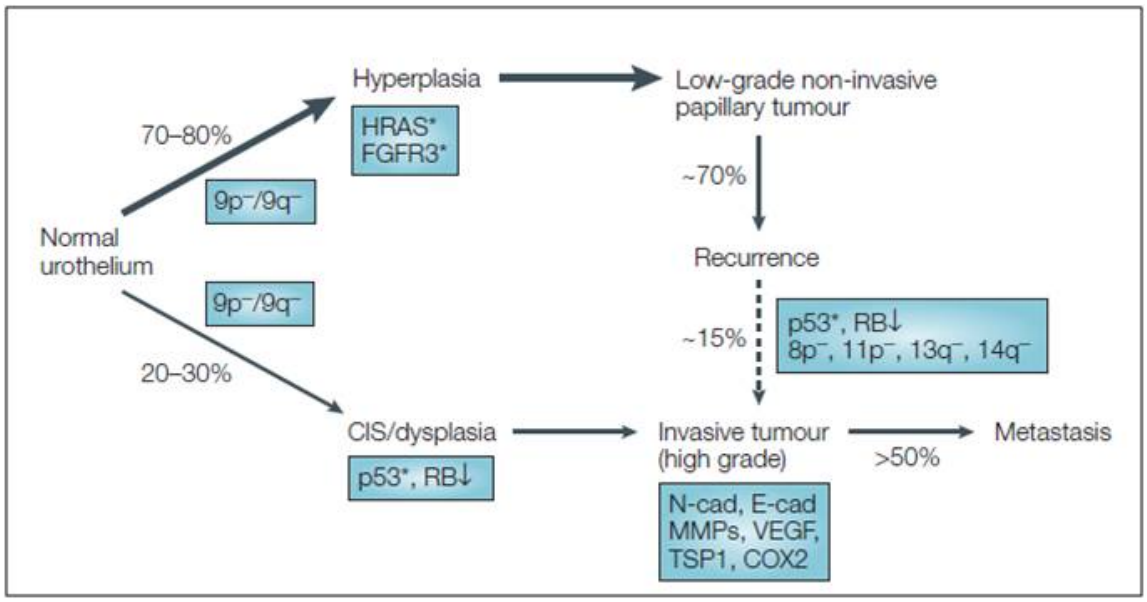


Figure 2.8.1: Important genetic and epigenetic defects that characterize the divergent pathways of urothelial tumorigenesis [86].

2.8.1. Molecular pathways in urothelial tumorigenesis

2.8.1.a. FGFR3 activation in non-muscle-invasive urothelial carcinomas

FGFR3 is a member of a structurally related family of receptor tyrosine kinases (FGFR1-4), which play important roles in cell proliferation, growth, differentiation, migration, and survival. Receptor tyrosine kinases are located in the cell membrane and consist of extracellular ligand-binding domains and an intracellular tyrosine kinase domain. Activation by their respective ligands induces kinase activation, which subsequently triggers intracellular signaling pathways associated with the aforementioned key cellular processes [87].

FGFR3 is activated by somatic mutations, which are thought to be the most frequent mutational events in low-grade urothelial carcinomas. Exons 7, 10 and 15 (Figure 2.8.2) represent putative hotspots, as most mutations have been detected within them in numerous studies employing human urothelial carcinomas [88-93]. *FGFR3* mutations have been detected in 68-77% of pTa bladder tumors [88-90], which indicates that they constitute primary genetic alterations occurring early in bladder tumor development [88]. *FGFR3* mutations have been positively associated with tumors of

not only lower stage (pTa), but also of lower grade [89-91, 94]. In a study analyzing urine sediment samples, the majority of mutations was detected in samples from patients who were later diagnosed with pTa tumors; it was also proposed that detection of *FGFR3* mutations can be used as a more sensitive or at least complementary means in the detection of low-grade, non-muscle invasive lesions [92]. A decreasing frequency of *FGFR3* mutations is consecutively noted with advancing tumor stage, as they have been detected in 16.8-31% of pT1 tumors [88-93] and in 5-16% of ≥ pT2 tumors [88-90]. Many of the mutations detected within the *FGFR3* gene are identical to those responsible for several autosomal dominant developmental and skeletal disorders, such as hypochondroplasia, achondroplasia and thanatophoric dysplasia [95].

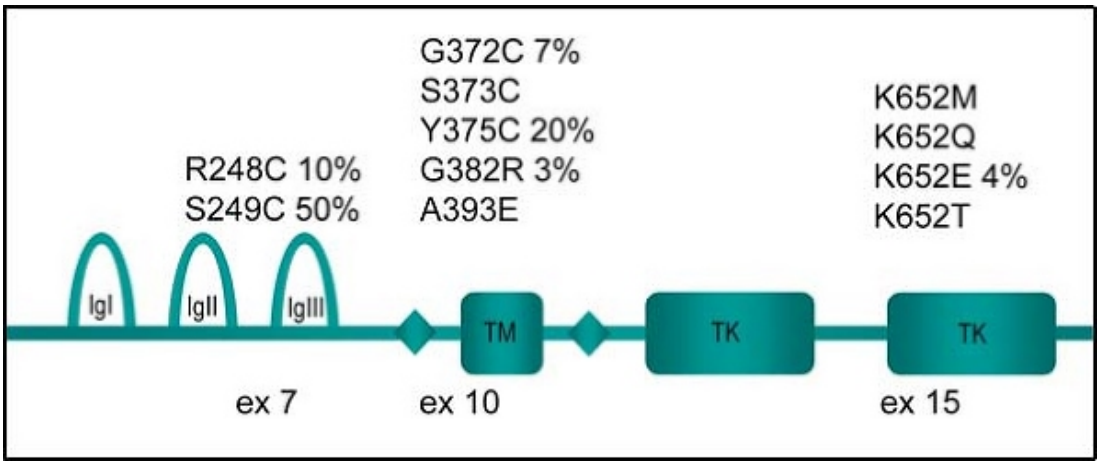


Figure 2.8.2: FGFR3 mutations identified in bladder cancer. Percentages show the relative frequency of the more common mutations. (IgI, IgII, IgIII, immunoglobulin-like domains; TM, transmembrane domain; TK, tyrosine kinase domain)

Activation of *FGFR3* is also mediated by protein overexpression. Gómez-Román et al reported marked FGFR3 mRNA and protein overexpression, especially in pTa and pT1 urothelial carcinomas. FGFR3 protein was also detected by immunohistochemistry in most low-grade, non-muscle-invasive tumors and in 50% of the high-grade, muscle-invasive ones. Interestingly, inhibition of cell growth was observed when anti-FGFR3

antibodies were used in transitional carcinoma cell lines, pointing out the crucial role FGFR3 holds in urothelial tumorigenesis [96]. In another study, higher FGFR3 protein levels were measured in non-muscle-invasive lesions and were significantly associated with low-grade tumors, whereas loss of FGFR3 expression was reported with advanced disease stage. Notably, it was shown that the protein is predominantly expressed in tumors harboring *FGFR3* mutations [94].

An additional mechanism of *FGFR3* activation in urothelial tumors has been recognized in a recent study employing 43 bladder tumor cell lines and 32 tumor samples. FGFR3 fusion protiens were identified in 4 cell lines and 2 tissue samples due to a reciprocal translocation and chromosomal re-arrangements resulting in constitutively activated FGFR3 fusion genes. As most of the tumors screened did not harbor *FGFR3* point mutations, Williams et al proposed that this mechanism may be more common in urothelial carcinomas with high levels of wild-type FGFR3 protein [97].

2.8.1.b. p53 and pRb inactivation in muscle-invasive urothelial carcinomas

While FGFR3 activation is generally accepted as the key molecular alteration underlying the development of low-grade, non muscle-invasive urothelial carcinomas, malignant lesions of higher stage and grade are thought to arise via tumor-suppressor gene inactivation, mainly of p53 and pRb.

p53 is a tumor suppressor gene involved in cell cycle regulation, induction of apoptosis and angiogenesis regulation [98]. Wild-type p53 protein has long been designated as “the guardian of the genome” and p53-deficient mice are known to have a strong predisposition to cancer [99]. *p53* mutations are thought to arise first in one allele and are followed by loss of the second, thus leading to loss of p53 normal function [86].

Studies employing human bladder carcinomas have identified *p53* mutations in 47% of ≥ pT2 tumors [88] and in 58% of non-muscle-invasive yet poorly differentiated TCCs [93]. Another study on 140 urothelial tumors showed that *p53* mutations are gathered in the DNA-binding domain of the gene, which is crucial for the protein folding and its interaction with DNA (Figure 2.8.3) [100]. Hernández et al also demonstrated that 30%

of the tumors harboring p53 mutations retain one wild-type allele [93]. Moreover, p53 mutations have been associated with disease recurrence and were almost twice more frequent in patients with lymph node-positive than in those with organ confined disease [101]. Nuclear accumulation of p53 protein, which is a prerequisite for its detection by immunohistochemistry (IHC), has been linked to mutations within the gene, especially missense ones [93, 101]. In many studies, p53 overexpression has been associated with increased stage and grade of bladder tumors [89, 91, 101]. In a recent analysis of 3,421 patients, Goebell et al found that p53 expression can be predictive of an unfavorable disease outcome in ≥ pT1 tumors [102].

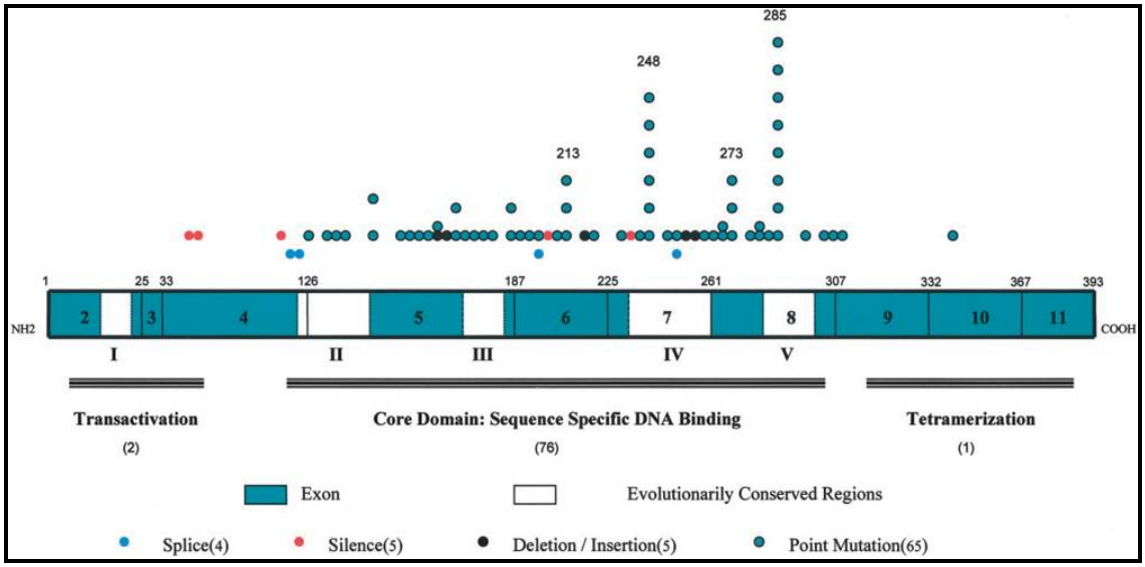


Figure 2.8.3: Distribution of *TP53* mutations. The majority of mutations are clustered in the core domain. Only 3 of 79 mutations are detected in the transactivation domain, including two silent mutations and one exon 10 point mutation affecting the tetramerization domain [100].

The *RB1* gene (Figure 2.8.4) encodes a protein (pRb) with multiple roles in development, cell differentiation, cell-cycle arrest and apoptosis and is another tumor-suppressor frequently disrupted in muscle-invasive bladder tumors [86]. Germline mutations in the *RB1* gene are the cause of retinoblastoma, which is a tumor of the

retina affecting young individuals. Retinoblastoma patients who have survived after treatment are at a higher risk of developing epithelial tumors later in their lives [103].

Loss of heterozygosity (LOH) has long been identified as a mechanism of *RB1* inactivation in bladder carcinomas. Cairns et al showed that *RB1* LOH not only correlates with tumor grade and is more frequent in muscle-invasive lesions, but may also be linked to rapid progression of non-muscle-invasive lesions to invasive ones [104]. Interestingly, loss of pRb expression was associated with radiosensitivity of tumors and with prolonged relapse-free survival in patients with muscle-invasive lesions [105]. Altered pRb expression, i.e. absent or elevated levels as detected by IHC, has been observed in ≥ pT2 tumors [106] and has been associated with both stage and grade in muscle-invasive TCCs [107]. Altered pRb has also been associated with an increased risk for recurrence and progression in pT1 tumors of grade 3 [108]. Moreover, independent studies have shown that muscle-invasive tumors harboring alterations in both p53 and pRb expression are more likely to recur or to cause death when compared with tumors with normal p53 and pRb expression or alterations in p53 or pRb alone [106, 107, 109]. These findings point out that p53 and pRb may synergistically promote urothelial tumor progression.

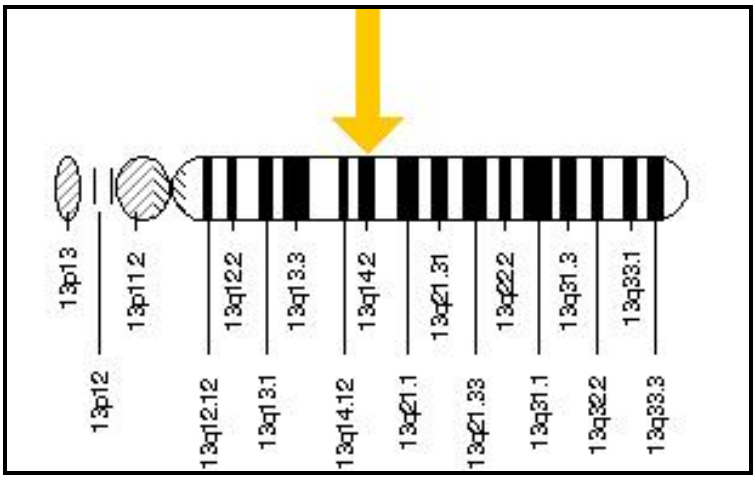


Figure 2.8.4: Location of the *RB1* gene on the lower arm of chromosome 13.

As pRb is functionally inactivated in tumors otherwise expressing high levels of it, alternative mechanisms for this disruption were investigated. In a study by Chaterjee et al, it was shown that constitutive inactivation of pRb function in bladder tumors can result from hyperphosphorylation. pRb hyperphosphorylation was significantly associated with high levels of the protein as it was evaluated by IHC. Also, tumors with increased pRb reactivity were found to express its hyperphosphorylated form, whereas underphosphorylated pRb was more frequently detected in the tumors with low or moderate protein expression [110].

Alterations in *FGFR3* and *p53* most probably comprise mutually exclusive events in the initiation of bladder tumorigenesis, as was demonstrated in previous studies [88, 89]. Bakkar et al rarely detected mutations in both *FGFR3* and *p53* in pT1-pT4 urothelial tumors [88]. In addition, the majority of urothelial tumors (staged pT1-pT4 as well) examined by van Rhijn et al were positive for either FGFR3 or p53 alone, whereas p53 overexpression was inversely related to FGFR3 mutation [89]. Hernández et al reported a similar frequency of *p53* mutation in *FGFR3* wild-type and mutant tumors and proposed an independent distribution of the two mutational events, yet their analysis was restricted in high-grade pT1 tumors [93].

2.8.2. Activation of RAS family genes in bladder tumors

Genes of the RAS family (*HRAS*, *KRAS*, *NRAS*) encode proteins involved in differentiation, cell cycle control and cell growth regulation. RAS proteins are 21-kDa G proteins ($p21^{RAS}$ proteins) with intrinsic GTPase activity, which are found associated with the cell membrane. Upon interaction with tyrosine kinase receptors, RAS proteins can activate signal transduction pathways within the cell [111, 112].

Activation of *RAS* genes in human cancers is frequent and can result from gene mutations or overexpression. Mutations in the *HRAS* gene are clustered in codons 12, 13 and 61 and can result in *HRAS* constitutive activation, thus converting it into an

oncogene [112]. It has been suggested that activation of *RAS* genes may represent an initiating event in bladder tumorigenesis. Zhang et al found that activated HRAS can cause hyperplasia of the urothelium in mice and, ultimately, induce the formation of non-muscle-invasive papillary tumors, which have limited potential for progression [113]. Interestingly, it was demonstrated in another study that activated HRAS induces early-onset non-muscle-invasive bladder tumors of either low or high grade in *p53* nullizygous mice [114].

HRAS mutations in human bladder tumors have been reported with strikingly varying frequencies (0-84%), which probably reflects technical differences between studies [86]. Earlier studies reported *HRAS* mutations in 0-6% of the tumors analyzed [115, 116]. However, later studies have identified *HRAS* codon 12 mutations in 84%, 46.7% and 30% of urothelial carcinomas [117, 118, 119]. These mutations have been associated with pTa and pT1 urothelial lesions [119]. Recently, Boulalas et al detected *HRAS* codon 12 mutations more frequently in non-muscle-invasive TCCs of the bladder but no *KRAS* and *NRAS* codon 12 and 13 mutations [119]. In the same study, KRAS and NRAS were found to be overexpressed in 50% of the tumors and a significant association of NRAS overexpression with poorly differentiated tumors was identified [119].

2.8.3. The role of ERBB family of receptors in bladder tumors

Besides *FGFR3*, the genes of the *ERBB* family have been implicated in the development of bladder malignancies. These genes code for RTKs, mainly ErbB1 (Epidermal Growth Factor Receptor, EGFR), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). Almost twenty ligands bind to these receptors, ultimately leading to activation of intracellular regulatory pathways which orchestrate cell proliferation, differentiation, migration and adhesion [120, 121]. The role of *ERBB* genes has been extensively studied in breast carcinomas, in which particularly *ERBB2* amplification has been associated with poor prognosis [121].

Earlier studies have demonstrated that EGFR expression is a rare event in non-muscle-invasive, papillary urothelial tumors, yet its overexpression is associated with their progression [122]. A later study employing 245 patients with TCCs of the bladder showed activation of *ERBB* genes and frequent combined expression of 2 or more of them in the tumors. ErbB2 and ErbB3 expression correlated with first tumor recurrence, whereas co-expression of EGFR-ErbB2-ErbB3 was significantly associated with second tumor recurrence. ErbB2 was also recognized as an important predictor for patient survival, whether co-expressed with EGFR or ErbB3 or expressed alone [123]. Memon et al later reported significant ErbB3 (HER3) downregulation in non-papillary, high-grade, ≥ pT2 urothelial tumors. In addition, they found that higher mRNA levels of HER4 and its ligand HRG4 correlated with better patient survival [124].

2.8.4. Molecules involved in angiogenesis and invasion of urothelial tumors

Angiogenesis, i.e. the formation of new blood vessels, is essential for cell function and survival, as it ensures oxygen and nutrient supply. A multistep process, it depends on the balance of pro-angiogenic factors and inhibitors, as well as on interactions with the extracellular matrix. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are established angiogenic inducers, whereas thrombospondin-1 (TSP-1) is well known to inhibit angiogenesis [85].

Increased VEGF expression in human urothelial tumors has been noted in several studies [125-129] and has been associated with poorly differentiated, muscle-invasive tumor phenotypes [125, 126]. Kopparapu et al also reported that human urothelial tumors and cell lines express higher levels of VEGF and its receptors VEGFR1 and VEGFR2, with VEGFR1 exhibiting its highest levels in grade 3 TCCs [129]. In another study, VEGFR2 elevated expression correlated with advanced tumor stage and increased invasive potential [128]. Apart from VEGF and its receptors, basic fibroblast growth factor (bFGF), another potent angiogenesis inducer, is also up-regulated in muscle-invasive bladder tumors [126, 127] and predicts disease recurrence [127].

Moreover, Shariat et al found that 63% of the tumors analyzed expressed low levels of TSP-1 and identified a significant correlation between TSP-1 down-regulation, recurrence and cancer specific mortality [127]. Donmez et al had previously reported decreased TSP-1 immunoreactivity in high-grade and invasive lesions [125]. Significantly increased TSP-1 expression has been noted in well to moderately differentiated urothelial carcinomas [130]. Beecken et al recently observed that TSP-1 expression was limited in dysplastic urothelial cells and well differentiated papillary tumors. On the contrary, cell lines staged pT2 and pT3 and of moderate to poor differentiation did not express TSP-1. Of them, the most aggressive one had also lost expression of endostatin, which also inhibits angiogenesis [131].

The aggressiveness of malignant cells is further determined by their ability to invade adjacent normal tissues and to spread to distant ones via a multifaceted mechanism termed the ‘‘epithelial-mesenchymal transition’’ (EMT) [83].

Cadherins are a family of transmembrane glycoproteins involved in cell-cell adhesion and the so-called “cadherin switch” (loss of E-cadherin and gain of N-cadherin expression) appears to be of importance in the process of EMT [132].

Garcia del Muro et al showed that decreased expression of E-cadherin not only is more frequent in high-grade, muscle-invasive urothelial carcinomas, but also predicts poor survival, especially in patients with invasive lesions [133]. In accordance with these results, Kashibuchi et al noted a decrease in 5-year survival rates with abnormal expression of E-cadherin and α-, β- and γ-catenins, which are encountered in complexes with cadherin molecules. They also found a significant correlation of E-cadherin down-regulation with advanced tumor grade [134]. The latter finding was later confirmed by Jäger et al, who also detected significantly lower N- and P-cadherin levels in malignant urothelial tissues when comparing them to normal bladder mucosae. In the same study, P-cadherin was up-regulated in pTa-pT1 tumors and was identified as a favorable prognostic factor of cancer-related survival [132]. N-cadherin, which has an established role in the promotion of urothelial tumor cell invasion [135], was significantly overexpressed in muscle-invasive carcinomas of high grade and correlated with poor survival [132].

Matrix metalloproteinases (MMPs), a family of endopeptidases capable of degrading the extracellular matrix (ECM), have also been implicated in urothelial tumor angiogenesis, invasion and metastasis [135]. Previous studies have identified an association between increased MMP-9 expression and high-grade, muscle-invasive bladder tumors [125, 126]. A pro-invasive role for MMP-9 was proposed in a study by Slaton et al, who demonstrated inhibition of *in vitro* invasion mediated by MMP-9 up-regulation and E-cadherin down-regulation in highly metastatic transitional cells treated with interferon-α. They also suggested that the MMP-9:E-cadherin ratio is a strong predictor of both disease aggressiveness and recurrence [136]. In a comprehensive study employing 169 urothelial carcinomas, Wallard et al analyzed the transcriptional levels of 23 MMPs (MMP-1-3 and MMP-7-28) and of four primary endogenous tissue inhibitors of the MMPs (TIMP-1-4). Apart from MMP-20, all members of the MMP family, along with their inhibitors, were expressed in low to very high levels. Also, advanced tumor grade significantly correlated with MMP-1, MMP-2, MMP-10-15 and MMP-28 [137].

2.8.5. Alterations in molecules involved in carcinogen metabolism

The glutathione-S-transferases are enzymes involved in the metabolism of environmental carcinogens, reactive oxygen species and chemotherapeutic agents. Glutathione-S-transferase M1 (GSTM1) participates in the detoxification of compounds found in tobacco smoke, such as PAHs and benzo(a)pyrene. Glutathione-S-transferase T1 (GSTT1) catalyzes the conjugation of harmful chemicals with glutathione to facilitate their removal [138]. The NAT1 and NAT2 enzymes catalyze acetylation of aromatic and heterocyclic amines, which are known bladder carcinogens, and are particularly active in the liver, gastrointestinal tract, and urinary bladder [20]. It has been suggested that individuals bearing the “slow acetylator” phenotype are more susceptible to bladder cancer than “moderate” or “rapid/fast acetylators” [86].

In a study by Srivastava et al, the G/G genotype of the GSTM1313 polymorphism was significantly associated with a high risk for bladder cancer and with reduced enzyme activity [139]. In another large study of 1,150 patients with TCCs of the bladder and

1,149 controls, the *GSTM* null genotype and the *NAT2* "slow acetylator" genotype were associated with an increased risk for bladder cancer [140]. The latter finding was particularly evident for cigarette smokers and was later confirmed in a systematic review by Sanderson et al, who also pointed out that rapid *NAT1* acetylation genotype may also contribute to the disease in smokers who are already "slow *NAT2* acetylators" [20].

2.8.6. Chromosomal abnormalities in urothelial tumors

Chromosomal abnormalities are frequently observed in many types of human malignancies. In bladder cancer, the most prevalent ones are deletions and amplifications, which often result in aneuploidy [86, 140].

The most frequent chromosomal alterations in urothelial carcinomas are losses of all or parts of the long arm of chromosome 9 [141, 142]. Five major regions (9q11–13, 9p22–23, 9q12–13, 9q21–22, and 9q34) are affected by deletions early in the formation of pTa tumors, suggesting that one or more tumor suppressor genes may be residing in these regions [143]. Of interest, Hartmann et al identified chromosome 9 deletions in 86% of *in situ* carcinomas and in 75% of dysplastic urothelial lesions, indicating that chromosome 9 deletions take place early in both pathways of urothelial malignant transformation (Figure 2.7) [144]. In a study by Baud et al, 48% of urothelial tumors exhibited LOH in the region 9p21, where the tumor suppressor *CDKN2A* is located [145]. Furthermore, LOH and/or FISH analysis has revealed deletions of the region 17p13.1, where *p53* resides, in 84% of CIS and 53% of dysplasias [144]. In addition, a significant association has been recognized between loss of 8p and poorly differentiated, muscle-invasive urothelial carcinomas. Of note, 17.2% of the tumors examined by Knowles et al harbored only one copy of chromosome 8 [146]. Finally, LOH of 15q has been reported in almost 40% of urothelial tumors. The role of the DNA repair gene *RAD51*, which is located at 15q15.1, in urothelial cancers remains to be elucidated [147].

2.8.7. Epigenetic alterations in urothelial tumors

Epigenetic alterations, such as DNA methylation and histone modification, are inherited reversible changes in gene function that occur without any underlying change in DNA sequence [83, 148]. The study of epigenetic alterations in human malignancies has gained increasing interest over the recent years.

DNA methylation, which is catalyzed by enzymes called DNA methyltransferases, involves the addition of a methyl group to the cytosine ring of the CpG dinucleotide. When taking place within a gene promoter, DNA methylation results in functional inactivation of the gene. The most well characterized epigenetic change in urothelial tumors is aberrant methylation of tumor suppressor gene promoters [148].

Promoter methylation has been described to occur more frequently in malignant than in normal urothelium [149]. One of the first tumor suppressors in which methylation was investigated is *p16/CDKN2* [150]. This gene is located at 9p21 and encodes two alternatively spliced products, INK4A and ARF, which are involved in cell cycle arrest [86]. Gonzalez-Zulueta noted that methylation of 5´ CpG island of *p16/CDKN2* resulted in gene silencing and suggested that this mechanism accounts for *p16/CDKN2* functional inactivation in TCCs of the bladder [150]. Yates er al later identified E-cadherin methylation as an independent prognostic factor of disease progression. Along with E-cadherin, methylation of *TNFRSF25*, *RASSF1α* and *APC* was significantly associated with tumor progression [149]. In another study, frequent E-cadherin methylation was recognized and correlated with tumor stage [151]. Moreover, methylation of *RASSF1α*, which constitutes a potential tumor suppressor, has been associated with tumor stage, grade and progression [151, 152]. Many apoptosis-associated genes are also methylated in urothelial cancers. Friedrich et al reported significantly elevated methylation levels of *DAPK*, *BCL2*, *TERT* and *TNFRSF25* in malignant urothelial tissues and identified that methylation levels of *BCL2* correlated with tumor stage and grade [152]. Additionally, *DAPK* hypermethylation has been associated with high recurrence rates in non-muscle-invasive tumors [153].

3. THE P53 FAMILY GENES

In 1979, several independent groups identified a 53 kDa protein forming a complex with SV40 large T-antigen in SV40-transformed tumor cells, thus marking the discovery of the *TP53* gene [154].

TP53 is the most extensively studied tumor suppressor gene and plays an important role in the cell's response to stress conditions by activating genes involved in cell cycle arrest or programmed cell death [155, 156]. *TP53* mutations contribute significantly to the development and progression of human tumors [157].

In 1997, the *TP53* homologues *TP63* and *TP73* were identified [158, 159]. The high homology shared by the three genes both at the genomic and the protein level strongly supported the hypothesis that they are functionally related transcription factors and that *TP53* is no longer an orphan tumor suppressor. This initial notion has in part been overthrown by ongoing research documenting that all three genes code for a wide variety of protein isoforms with diverse -frequently even opposing- roles in development, proliferation, apoptosis and tumorigenesis [160].

3.1. Structure of the p53 family genes

3.1.1. Structure of the *TP53* gene

The human *TP53* gene is mapped on 17p13.1 and is composed of 19,200bp spanning over 11 exons (GenBank Accession Number: NC_000017.11).

The full-length 393-residue p53 protein consists of the N-terminus, the central core and the C-terminus (Figure 3.1.1).

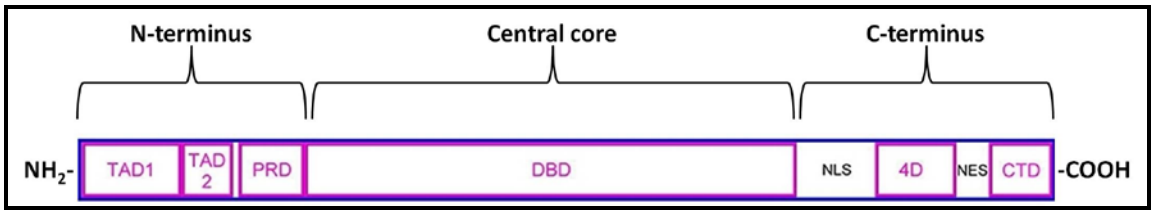


Figure 3.1.1: Functional domains of the human wild-type p53 protein [162].

The transactivation domain (TAD) is divided into two subdomains with complementary functions (TAD1 and TAD2) (Figure 3.1.2A). The main domain (TAD1, residues 1-41) is required for the generic transactivation functions and also contains the binding site for Mdm2, which is the main regulator of p53 protein stability. On the other hand, TAD2 (residues 42-63) is responsible for the selection of specific transactivation targets [161]. The transactivation domains are located on the N-terminus along with a Src homology 3-like (SH3) proline-rich domain, which interacts with proteins preventing p53 degradation and is involved in the regulation of apoptosis. The DNA-binding domain (DBD) (Figure 3.1.2B) is crucial for interactions between p53 and its target genes [162]. The majority of mutations are assumed to take place in exons 5-8, which encode the DBD [163]. The tetramerization domain (also termed the oligomerization domain, OD) (Figure 3.1.2C) is located in the C-terminus, along with nuclear localization and export signals (NLS and NES, respectively). The C-terminus itself has a non-specific DNA-binding activity and is thought to participate in p53-mediated repair of DNA damage and DNA and RNA re-annealing [161]. The C-terminal regulatory domain, particularly at its very end, consists of a high level of basic amino acids that are thought to regulate the core DNA-binding domain, although the precise mechanism of this remains elusive [161, 162].

As of recently, *TP53* structure was believed to be simple. Yet, it has been shown that *TP53* can produce at least twelve protein variants by alternative splicing, alternative initiation of translation and usage of an alternative promoter [164, 165].

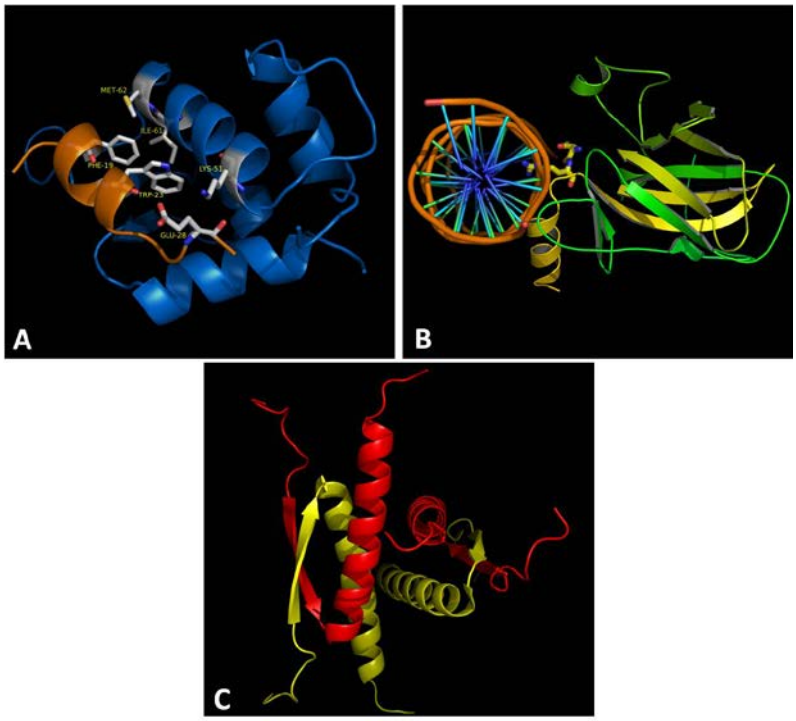


Figure 3.1.2: Structures of **(A)** p53 TAD1 domain, residues 17-29 (orange), bound to Mdm2 (blue), **(B)** p53 core domain bound to DNA (view through helical axis) and **(C)** p53 oligomerization domain (OD) tetramer, residues 319 to 359.

The Δ40p53 isoform is truncated of the first 40 amino-acids and is generated by either alternative splicing of intron 2 or by alternative initiation of translation (Figure 3.1.3A). The Δ40p53 isoform has been shown to use a second TAD to activate gene expression and is a dominant negative inhibitor of wild-type p53. The N-terminally truncated Δ133p53 and Δ160p53 isoforms are generated by usage of an internal promoter in intron 4. Due to alternative initiation of translation, the translation of Δ133p53 is initiated at codon 133, while that of Δ160p53 at codon 160. Therefore, those variants lack the transactivation domain, as well as a part of the DNA-binding domain [164, 165]. Alternative splicing in intron 9 creates p53α (full-length), p53β and p53γ, with the last two lacking the oligomerization domain (Figure 3.1.3A) [164, 165]. Therefore, twelve p53 protein variants are generated by alternative splicing, alternative initiation of translation and usage of an alternative promoter: p53α, p53β, p53γ, Δ133p53α, Δ133p53β, Δ133p53γ, Δ40p53α, Δ40p53β, Δ40p53γ, Δ160p53α, Δ160p53β and Δ160p53γ (Figure 3.1.3B) [165].

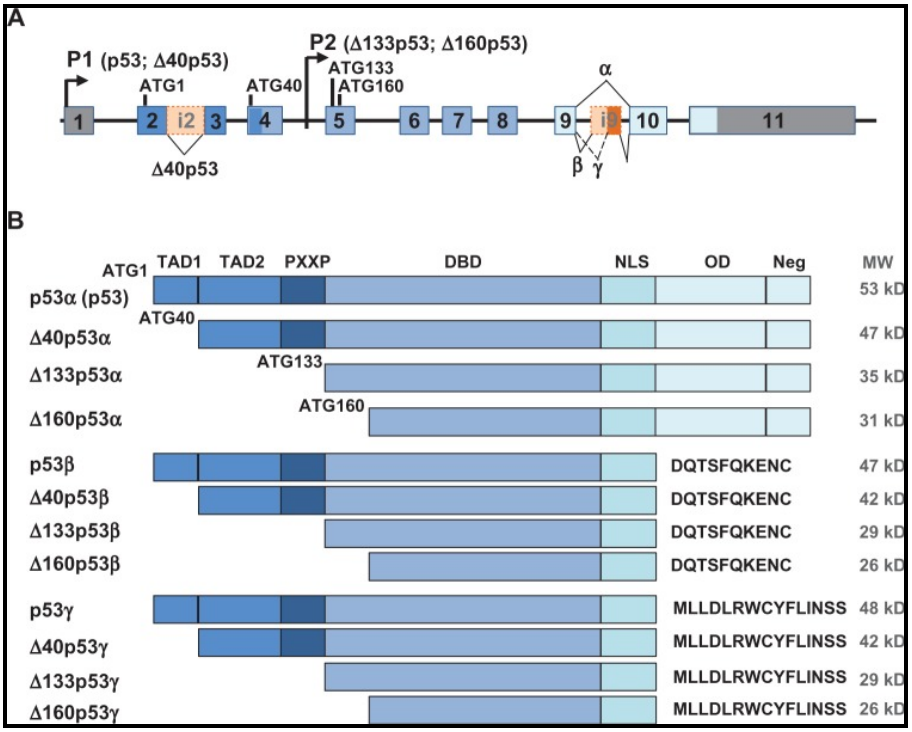


Figure 3.1.3: A. The human *TP53* gene structure: alternative promoters (P1 and P2), sites of alternative initiation of translation (ATG1, ATG40, ATG133 and ATG160) and alternative splicing (α, β, γ) are indicated. **B.** p53 protein isoforms [165].

3.1.2. Structure of the *TP63* gene

The human *TP63* is mapped on 3q27 and is composed of 15 exons spanning over 270,000bp (GenBank Accession Number NC_000003.12).

TP63 encodes at least six open reading frames from the usage of two different promoters in combination with three alternatively spliced C-terminal ends. The TAp63 isoforms are produced by the 5´ promoter upstream of exon 1 and retain the transactivation (TA) domain. The N-terminally truncated ΔNp63 isoforms are generated by usage of an alternative promoter in intron 3 and a second transcription initiation codon and lack the TA domain. Alternative splicing at the C-terminus gives rise to:

- the p63α isoforms, which contain all exons (11-14) of the C-terminus,
- the p63β isoforms, which lack exon 13 and

- the p63γ isoforms, which lack exons 11-14 (Figure 3.1.4a).

Altogether, *TP63* codes for six different protein isoforms: TAp63α (full-length), TAp63β, TAp63γ, ΔNp63α, ΔNp63β and ΔNp63γ (Figure 3.1.4b). ΔNp63 isoforms can exert dominant-negative effects over p53, TAp63 and TAp73 by either competing with them for DNA binding sites or by direct protein-protein interactions [164].

The oligomerization domain (OD) is conserved among all p53 family members and is responsible for the formation of homo-oligomers. Initial studies suggested that the OD domains of p63 and p73 can fold to form stable homo-oligomers. However, the extent of hetero-oligomerization between p63 and p73 was much less efficient. In addition, it was demonstrated that the p53 OD domain does not interact with those of p63 and p73 to form hetero-oligomers, even when p53 is in 15-fold excess. Although the p63-p73 interactions were found to be weak, it was shown that a p73 dominant negative mutant could clearly inhibit the transactivational activity of p63 [166].

The TAp63α and ΔNp63α isoforms also contain an additional region in their C-termini, known as sterile alpha motif (SAM) domain, which is not found in p53. This domain is responsible for protein-protein interactions and is thought to be have a role in developmental processes, as well as in apoptosis, transcriptional transactivation, focal adhesion, chromatin remodeling, receptor tyrosine kinase signaling and SUMOylation [167].

3.1.3. Structure of the *TP73* gene

The human *TP73* is mapped on 1p36.3 and is composed of 15 exons spanning over 80,000 bp (GenBank Accession Number NC_000001.11).

TP73 encodes at least 35 mRNA variants, which can theoretically be translated into 29 different protein isoforms. The TAp73 isoforms are produced by the promoter located upstream of exon 1 and retain the transactivation (TA) domain. The N-terminally truncated ΔNp73 isoforms, which lack the TA domain, are generated by usage of an alternative promoter in intron 3. Alternative splicing at the N-terminus produces:

- the p73 isoforms, which contain both exons (2-3) of the TA domain,
- the ex2p73 isoforms, which lack only exon 2 of the TA domain,
- the ex2/3p73 isoforms, which lack both exons (2-3) of the TA domain and
- the ΔN´p73 isoform, which contains exons 2, 3 and 3´ (Figure 3.1.5a) [164].

The ex2p73 isoforms have lost the conserved sequence of the TA domain, yet still contain part of it. The ex2/3p73 isoforms have entirely lost the TA domain and are initiated from exon 4. The ΔN´p73 isoform, which is often overexpressed in tumors, contains the normal ATG in exon 2 and a stop codon in exon 3´. The protein encoded by ΔN´p73 has so far not been described, yet it could be a short protein containing only the TA domain [164].

Extensive alternative splicing at the C-terminus gives rise to at least seven variants:

- the p73α isoforms, which contain all exons (11-14) of the C-terminus,
- the p73β isoforms, which lack exon 13,
- the p73γ isoforms, which lack exon 11,
- the p73δ isoforms, which lack exons 11-13,
- the p73ε isoforms, which lack exons 11 and 13,
- the p73ζ isoforms, which lack exons 11 and 12,
- the p73η isoforms, which lack exon 14 (Figure 3.1.4β) [164].

In contrast to p63, p73 isoforms can be initiated from different translation initiation sites and therefore contain different parts of the N-terminus, suggesting that they can engage in distinct protein interaction and specific activities [164].

Overall, *TP73* codes for 29 different protein isoforms: TAp73α (full-length), TAp73β, TAp73γ, TAp73δ, TAp73ε, TAp73ζ, TAp73η, ΔNp73α, ΔNp73β, ΔNp73γ, ΔNp73δ, ΔNp73ε, ΔNp73ζ, ΔNp73η, ΔN´p73, ex2p73α, ex2p73β, ex2p73γ, ex2p73δ, ex2p73ε, ex2p73ζ, ex2p73η, ex2/3p73α, ex2/3p73β, ex2/3p73γ, ex2/3p73δ, ex2/3p73ε,

ex2/3p73ζ and ex2/3p73η (Figure 3.1.4b). ΔNp73 isoforms are thought to have dominant negative activity like their ΔNp63 counterparts, and ΔNp73β and ΔNp73γ exhibit higher transactivation activity than ΔNp73α [164].

Although the SAM domains of some proteins have been shown to form homo- or hetero-dimers, this is not the case for the SAM domains of *TP73* and *TP63*, indicating that they interact with other proteins rather than with themselves or with proteins of the p53 family [165].

Interestingly, the C-terminal region of *TP73* has been described to contain an intrinsic transactivation activity, which is abrogated by two mutations detected in neuroblastoma [167].

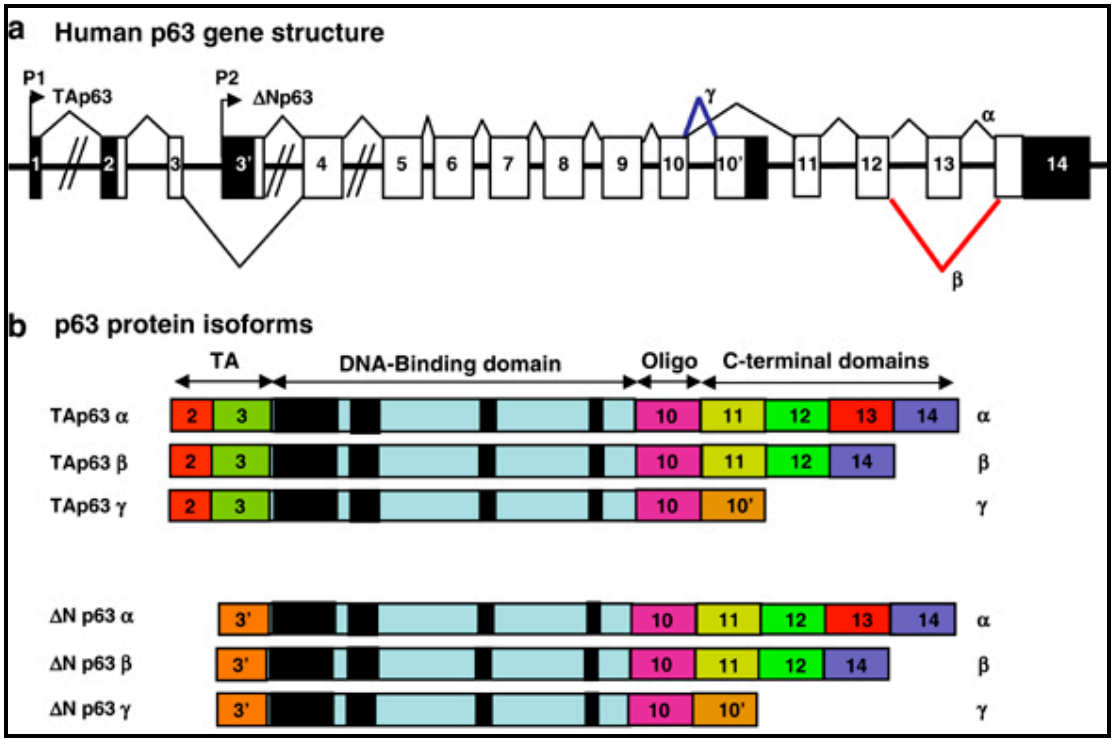


Figure 3.1.4: a. The human *TP63* gene structure: alternative promoters (P1 and P2) and alternative splicing (α, β and γ) are indicated. **b.** p63 protein isoforms. Numbers indicate the exons encoding p63 protein isoforms [164].

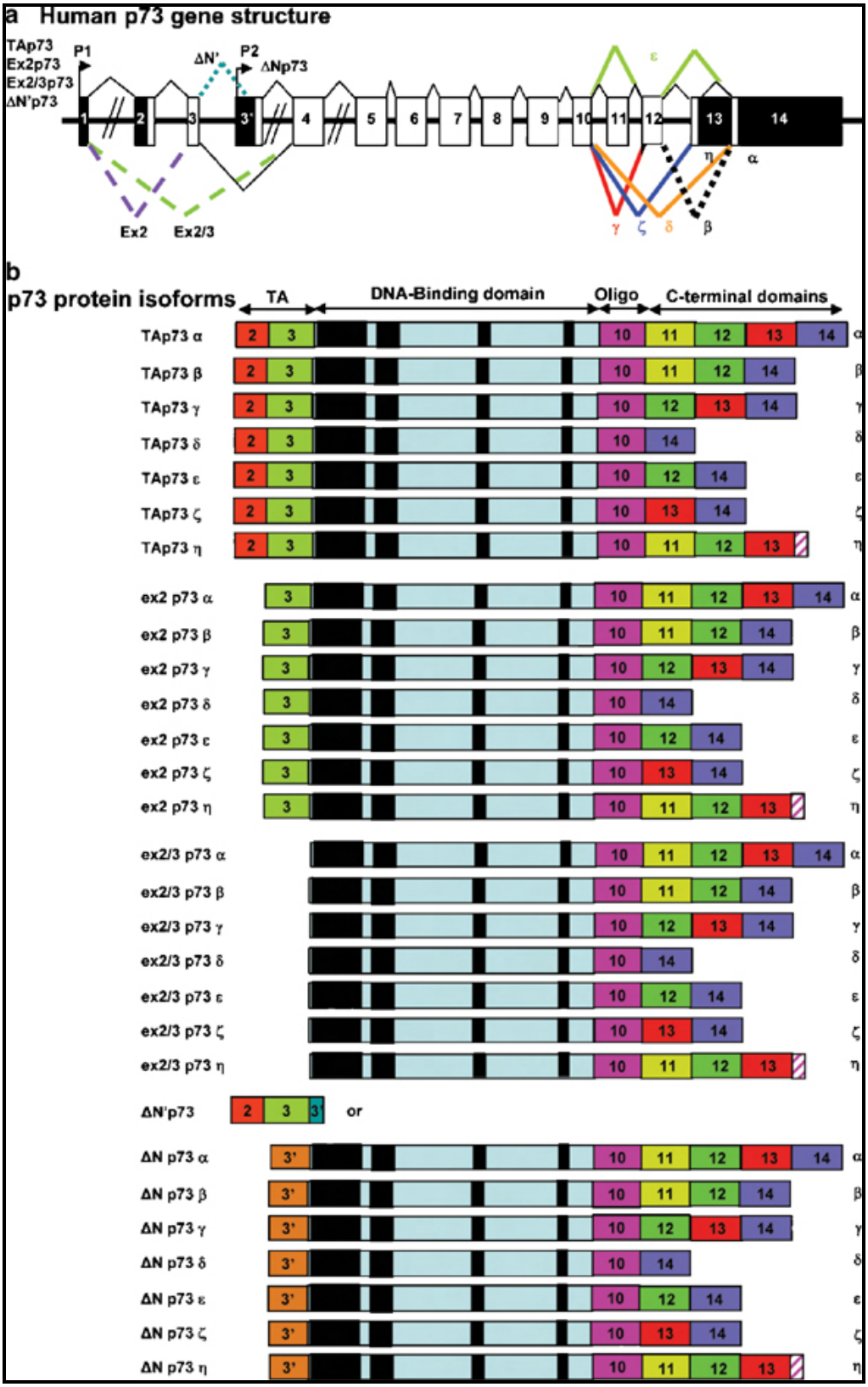


Figure 3.1.5: a. The human *TP73* gene structure: alternative promoters (P1 and P2) and alternative splicing (α, β, γ, δ, ε, ζ and η) are indicated. **b.** p73 protein isoforms. Numbers indicate the exons encoding p73 protein isoforms [164].

3.2. Mutations of the p53 family genes

3.2.1. Mutations of the *TP53* gene

Germline *TP53* mutations are the cause of the Li-Fraumeni or Li-Fraumeni-like syndrome, which is associated with a strong predisposition to certain types of malignancies, such as bone and soft-tissue sarcomas, premenopausal breast carcinoma, brain tumors, adrenocortical carcinoma, leukemias, melanoma, Wilms' tumor, and lung, gastric and pancreatic carcinomas [169].

Somatic mutations within the *TP53* sequence or LOH in 17p13.1 are the primary causes of p53 inactivation in 50% of sporadic human malignancies [157].

Although most tumor suppressor genes are inactivated by frameshift or nonsense mutations, which result in absence of protein synthesis (or production of a truncated product), the majority of *TP53* alterations are missense mutations, followed by frameshift and nonsense mutations (Figure 3.2.1) [170]. These mutations mostly occur in sequences encoding the DNA-binding domain and can affect protein folding which is essential for proper function (Figure 3.1.5) [100, 163, 171]. Missense mutations increase the half-life of the p53 protein [157], which accumulates in the cell nucleus, but has impaired ability to interact with DNA [163]. So far, three types of p53 mutants have been described: (i) dominant negative mutants which inhibit the suppressive function of the residual wild-type p53 protein, (ii) loss of function mutants which lack suppressive function and (iii) and gain of function mutants which exhibit oncogenic properties by aberrant protein interactions or gene regulation [163]. Frameshift mutations, accounting for 11% of all *TP53* mutations, have been shown to lead to total absence of p53 protein expression [171].

As of 2008, *TP53* mutations had been detected in 90% of ovarian carcinomas, 50-80% of lung carcinomas (depending on the subtype), 65-75% of esophageal carcinomas, 40-60% of colorectal carcinomas, 50% of bladder tumors, 30-45% of stomach carcinomas, 30-40% of liver carcinomas (depending on the etiology of the tumor), 30-40% of breast cancers (depending on the subtype), 30% of brain and other nervous system tumors, 10% of prostate cancers (with higher frequency in metastatic tumors) and in less than

5% of cervical carcinomas, in which they negatively correlate with human papilloma virus (HPV) infection. The frequency of *TP53* mutations in leukemias and non-Hodgkin lymphomas depends on the type of the malignancy, yet can reach 50% at disease relapse [171].

Codons 175, 245, 248, 273 and 282, all located in the sequence encoding the DBD, represent hot spots for mutation in human cancers (Figure 3.2.1) [157]. It has previously been shown that all *TP53* hot spot mutations result in loss of protein activity [171].

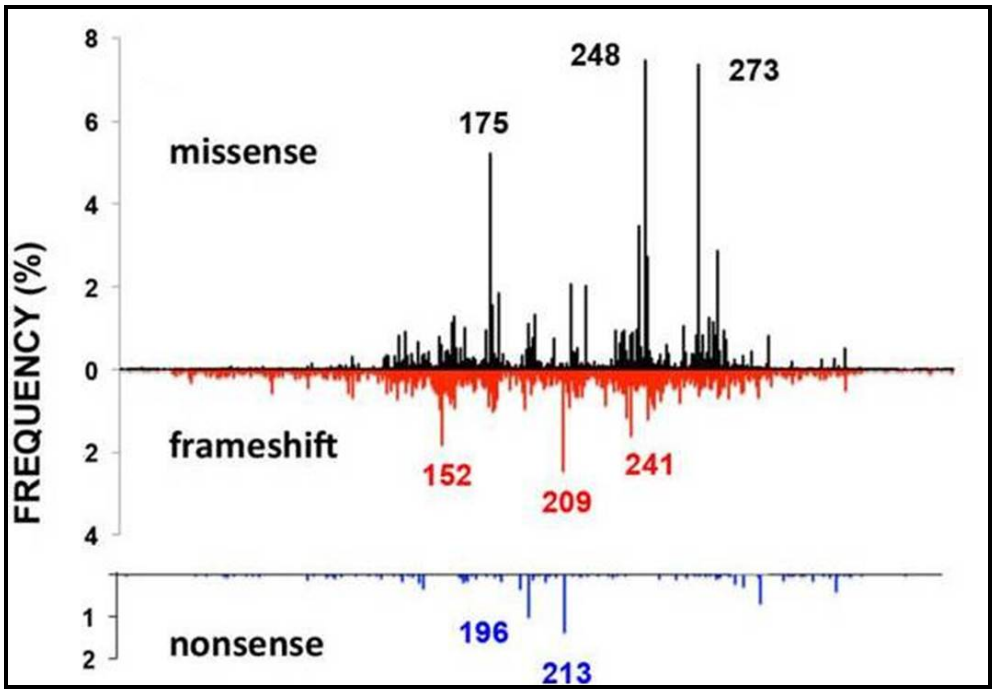


Figure 3.2.1: Distribution of *TP53* mutations in the TP53 protein [171].

In urothelial tumors, *TP53* mutations are more evenly distributed along the gene and occur at both CpG sites, such as codons 175, 248 and 273 and at non-CpG sites, such as codons 280 and 285. Of note, codons 280 and 285 are unique hot-spots for *TP53* mutations in bladder cancer and other urinary tract malignancies and are rarely mutated in other epithelial tumors. These distinguished mutational features of *TP53* in bladder cancer have been attributed to a urothelium-specific carcinogen, as is 4-aminobiphenyl [172].

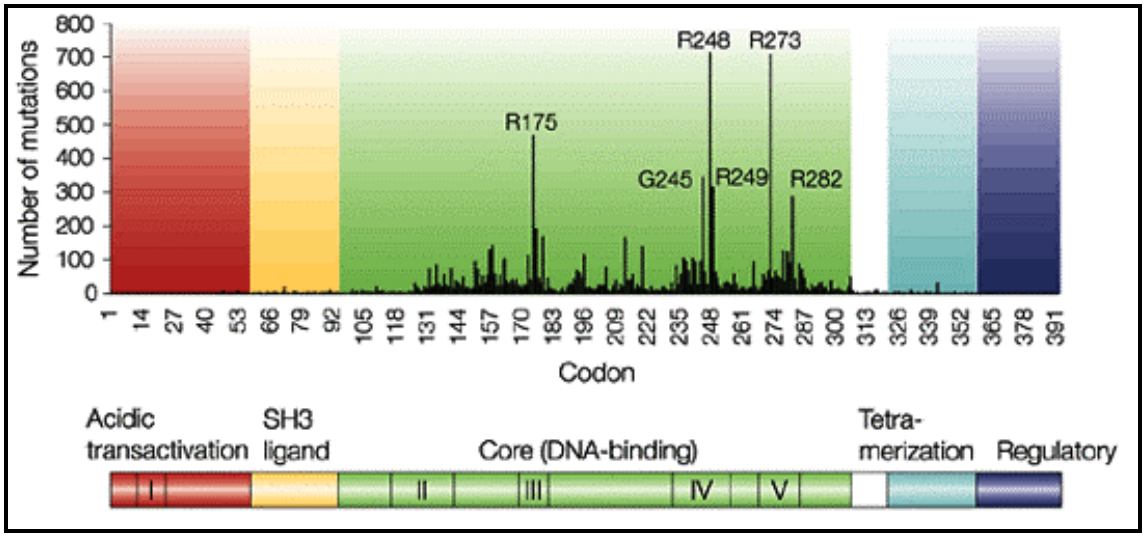


Figure 3.2.2: Histogram of p53 missense mutations, showing that 95% of them occur in the core domain. Residues R175, G245, R248, R273 and R282 are hot spots for mutation. Boxes I-V show the regions of highest sequence conservation [170].

p53 interaction with viral oncoproteins. Apart from mutation, inactivation of the p53 protein can occur by its interaction with viral oncoproteins, such as the SV40 large T-antigen, adenovirus proteins, HPV-16 E6 protein and the Hepatitis B virus (HBV) X antigen. It has been demonstrated that the HPV-16 E6 protein can induce the degradation of p53, whereas the Adenovirus E1B 55 K and E4orf6 proteins were shown to inhibit the transactivating function of p53 [167, 173].

3.2.2. Mutations of the *TP63* gene

Unlike *TP53*, *TP63* is rarely mutated in human cancers. Shortly after its discovery, Hagiwara et al performed mutational analyses on 54 human cell lines (colon cancers, non-small cell lung cancers, small cell lung cancers, breast cancers, hepatocellular carcinomas, hepatoblastoma, hepatoblastoma transfected by HBV, SV40 immortalized liver cells, pancreatic cancers, oral cancers, mesotheliomas, T-cell lymphoblastic leukemia, T-cell lymphoma, ovarian cancer, cervical cancer, esophageal cancer and glioblastoma) and identified one point mutation in exon 6 and another in exon 13, both resulting in amino-acid substitution [174]. Tani et al screened 44 lung cancer cell

lines and 45 primary lung cancers and found one frameshift mutation in exon 5 in one cell line of squamous origin [175]. The TAp63γ isoform was also screened for mutations in 80 non-small cell lung cancers (NSCLCs) and in 85 breast tumors. Three missense and one silent mutation were identified in the NSCLCs, whereas no mutations were identified in the breast tumors [176]. Another mutation analysis revealed two missense variants, the first in 1/14 primary lung cancers and the second in 1/6 head and neck squamous cell carcinoma (HNSCC) cell lines included in the study [177]. In a study employing 80 chronic myelogenous leukemia cases, Yamaguchi et al detected *TP63* mutations in 6 patients and noted that in four of them the mutations clustered in codons 151-170 of the DNA-binding domain [178]. It should be noted that, although in very low frequencies, *TP63* mutations are detected mostly in exons coding for the transactivation and DNA-binding domains.

On the other hand, heterozygous germline *TP63* mutations have been strongly associated with rare yet severe developmental disorders of autosomal dominant inheritance.

Celli et al recognized *TP63* germline mutations as the cause of the Ectrodactyly-Ectodermal dysplasia-Clefting (EEC) syndrome, which is characterized by the absence of one or more central digits in hands and/or feet (Figure 3.2.3), defects on structures of ectodermal origin (hair, teeth, nails, sweat glands) and cleft lip with or without palate. After screening 25 unrelated EEC patients, they identified 8 missense mutations in the DNA-binding domain of *TP63* and one frameshift mutation generating a premature stop codon in exon 13 of the C-terminus. All missense mutations resulted in amino-acid substitution and partially or completely abrogated DNA-binding ability of the p63 protein [179].

Ankyloblepharon-Ectodermal dysplasia-Clefting (AEC) syndrome, also named Hay-Wells syndrome, differs from EEC in that patients have minimal or no limb malformation. Instead, AEC patients have partial or complete fusion of the eyelids. Mutation analysis in eight families with the clinical features of the AEC syndrome revealed single nucleotide transitions in exon 13, which result in amino-acid substitutions in the SAM domain, in all individuals examined. Several mutations were

found to be *de novo*, which further supports their strong contribution to the AEC syndrome. The mutations affect only the TAp63α and ΔNp63α isoforms, which retain exon 13, suggesting that they have distinct biological activities to the other p63 isoforms [180].

Limb-Mammary syndrome (LMS) is characterized by ectrodactyly, mammary gland and nipple hypoplasia, and cleft palate. This condition has been associated with frameshift mutations in exons 13 and 14, with the former resulting in p63 proteins lacking the SAM domain [181].

The Acro-Dermato-Ungual-Lacrimal-Tooth (ADULT) syndrome differs from the EEC syndrome by the absence of facial clefting. Recently, two *TP63* mutations were identified in ADULT patients, one within the TA domain and another in the DNA-binding domain. The latter at codon 298 was reported to confer a gain of function on ΔNp63γ isoform, which exhibited exceptionally high transactivation activity when compared to the wild-type protein [182].

Finally, split hand/foot malformation (SHFM) has been associated with missense, nonsense and splice-site *TP63* mutations, occurring mostly in the DNA-binding domain. Patients have clefts on the hands and feet but no other features of the EEC syndrome. It is thought that the majority of SHFM cases is caused by mutations in other genes, since the number of patients with *TP63* mutations is small [181].

p63 interaction with viral oncoproteins. Shortly after the discovery of the *TP63* gene, it was shown that the p63 protein does not interact either with the HPV-18 E6 protein or with the SV40 large T-antigen [183].

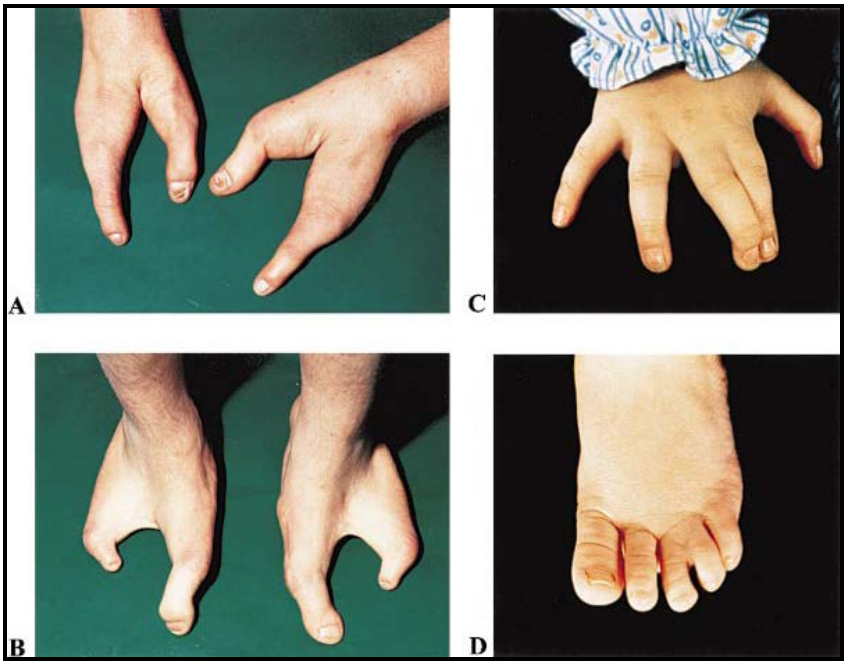


Figure 3.2.3: Limb defects in EEC syndrome patients with *TP63* mutations. Typical split-hand with dystrophic nails of the thumbs **(A)** and typical split-feet **(B)** from an EEC syndrome patient with a frameshift mutation in exon 13. **(C)** Cutaneous syndactyly of second and third fingers of the right hand and **(D)** partial syndactyly of the third and fourth toes of the left foot of a patient with a missense mutation in codon 304 [179].

3.2.3. Mutations of the *TP73* gene

The discovery of *TP73* was succeeded by intense efforts to detect potential mutations within its sequence that could possibly be associated with tumorigenesis. Yet, like *TP63*, *TP73* was rarely found to harbor mutations [184].

Upon its description, Kaghad et al screened the coding region of the *TP73* gene for mutations but found none in the 4 neuroblastoma cell lines analyzed [159]. Mutation analysis of 151 informative cases of neuroblastoma (NB) revealed one somatic *TP73* mutation in codon 405 in one tumor and one germline mutation in codon 425 in one tumor. Both are codons of the C-terminal domain, which has potential transactivational activity. In both cases, mutations resulted in amino-acid substitution and revealed a loss of function mutation [185]. After screening 30 NB tumors and 22 NB cell lines, Yang et al identified one silent *TP73* mutation in codon 336 in one

primary tumor [186]. In another analysis of 6 NB tumors expressing high levels of p73 mRNA, a C→T transition was identified in exon 2 in one tumor, along with four silent nucleotide changes in exons 9 and 14 in another tumor [187]. Kong et al found one T→C substitution in codon 173 in one NB cell without amino-acid substitution, after screening 32 primary NB tumors and 18 NB cell lines [188]. Analysis of 30 meningiomas revealed one missense mutation in codon 204 in one tumor, without amino-acid substitution [189]. In three other studies employing NB tumors and cell lines and one study employing meningioma tumors, no *TP73* mutations were identified [190-193]. In 1/65 nonastrocytic tumors analyzed by Alonso et al, one missense mutation was found in codon 291 in one tumor [194].

Five silent *TP73* mutations in 29 hereditary breast tumors but no mutations in 48 sporadic cases were identified by Alonso et al [195]. Two other analyses revealed no mutations in 164 breast cancers and 7 cell lines [196, 197].

TP73 is also rarely mutated in malignancies of the lung. Nicholson et al detected one single insertion in intron 3´ in 5/10 non-small cell lung cancers, which also exhibited loss of heterozygosity in *TP73* (discussed later) [198]. In another two studies, 82 lung tumors in total were examined for *TP73* mutations, but with no positive results [199, 200].

In a series of 22 hepatocellular carcinomas paired to normal liver tissues, only one tumor-specific 5-nucleotide deletion causing early truncation of the DNA-binding domain was identified [201]. Mihara et al detected no *TP73* mutation in 48 primary hepatocellular carcinomas matched to normal adjacent tissues [202].

Mutations of the *TP73* gene were not detected in three independent studies employing 87 esophageal SCCs and 62 adenocarcinomas in total [203-205].

In another three independent studies, 170 gastric adenocarcinomas, 68 multiple simultaneous gastric carcinomas, 5 gastric cell lines and 20 normal mucosae were screened in total, with no positive results for *TP73* mutations [206-208].

A total of 151 colorectal tumors were analyzed in two independent studies and no *TP73* mutations were identified [206, 209].

Faridoni-Laurens et al identified no mutations in 50 head and neck SCCs (HNSCCs) and 21 normal tissue samples [210].

TP73 mutations are not detected in gynecological malignancies, as was reported in two analyses of 20 invasive ovarian carcinomas [211] and 24 SCCs of the vulva [212].

Screening of the *TP73* region encoding the DNA-binding domain was performed in two independent studies on a total of 25 benign melanocytic nevi, 34 primary melanomas, 54 melanoma metastases and 9 melanoma cell lines, with no positive results for *TP73* mutations [213, 214].

Yokomizo et al analyzed 27 primary prostate tumors matched to their normal counterparts and found no tumor-specific *TP73* mutations [215]. Another mutation analysis performed on 106 prostate cancers identified no mutations as well [216].

Involvement of *TP73* mutations in malignant hematopoiesis is also rare. Kawano et al found no gene alterations in 43 hematopoietic and non-hematopoietic cell lines, which all expressed p73 mRNA [217]. Screening of exons 4,6,7 of the gene in 60 *de novo* acute myelogenous leukemia (AML) patients produced no positive results either [218].

Finally, Chi et al found no mutations after examining the coding region of the *TP73* mutations in 45 primary bladder carcinomas [219].

p73 interaction with viral oncoproteins. Although the HPV-16 E6 oncoprotein can induce degradation of p53, it has no such effect on p73α or p73β isoforms. Also, the SV40 large T-antigen and the Adenovirus E1B 55 K protein do not interact with p73α or p73β. As with p53, the Adenovirus E4orf6 and the human T-cell lymphotropic virus 1 (HTLV1) Tax proteins are capable of inactivating p73α and p73β isoforms by binding to their C-terminal regions [167, 184]. It has also been shown that the Hepatitis C virus (HCV) core protein binds to the C-termini of p73α and p73β (aa 321-353) and can modulate transcription from p53/p73 target promoters, even at low concentrations [220].

3.2.3.a. Loss of heterozygosity of the *TP73* gene

The *TP73* gene is mapped on 1p36, a chromosomal region undergoing frequent loss of heterozygosity (LOH) in neuroblastoma and other human tumors [159]. Indeed, incidence of 1p36 LOH has been reported in 15-35% of informative NB cases [185, 186, 192]. Ichimiya et al also identified a significant correlation of 1p36 LOH with sporadic disease, *N-myc* amplification and advanced disease stage [185]. In addition, 1p36 LOH was detected in approximately 50% of primary and metastatic meningiomas analyzed by Alonso et al [194].

In breast cancers, *TP73* LOH has been detected at frequencies ranging from 13% to at least 27% [196, 221, 222]. In a cohort of 193 breast carcinoma patients, *TP73* LOH was significantly associated with higher tumor grade and advanced stage, with peritumoral vessel involvement and with tumors exhibiting lymph node metastases [221]. Also, Ahomadegbe et al reported significantly higher *TP73* LOH frequency in inflammatory breast carcinomas (IBC), which are characterized by very poor prognosis, when compared to their counterparts with no inflammatory symptoms (NBC) [223].

LOH affecting the *TP73* locus has been detected in 18-40% of a total of 105 hepatocellular carcinomas [201, 202, 224] and in 30% of 22 intrahepatic cholangiocarcinomas analyzed [225]. Momoi et al noted higher 1p36 LOH frequency in cholangiocarcinomas of advanced stage, as well in large tumors without lymph node metastasis [225].

In malignancies of the gastrointestinal tract, *TP73* LOH has been reported at varying frequencies of 8-64.3% in esophageal tumors [203-205] and in 37.5% of gastric adenocarcinomas. Gastric tumors with *TP73* LOH showed abnormal p73 mRNA expression and specific histologic features of the faveolar epithelial phenotype [206]. Sunahara et al detected *TP73* LOH in 17% of the colorectal tumors examined [209].

In lung carcinomas, *TP73* LOH has been detected in 20.8% and 42.3% of the tumors examined [198, 200]. Nomoto et al noted higher frequency of *TP73* LOH in SCCs of the lung when compared to adenocarcinomas [200].

Loss of heterozygosity in 1p36 is quite frequent in ovarian carcinomas and has been detected in 8.3-50% of a total of 98 tumors analyzed in four independent studies [211, 226-228].

Finally, 1p36 LOH has been reported in 5.3% of prostate carcinomas [216] and in 28.1% of parathyroid adenomas [229].

So far and to our knowledge, *TP73* LOH has not been reported in urinary bladder tumors.

3.2.3.b. Imprinting of the *TP73* gene in human tumors and normal tissues

Imprinted genes are those expressed in a parent-of-origin specific pattern and monoallelic expression constitutes evidence for imprinting of a gene. Prior to the discovery of *TP73*, evidence for imprinting at the distal region of the short arm of chromosome 1 in malignancies emerged from studies of neuroblastoma. It was reported that the lost 1p36 alleles were more frequently of maternal origin, especially in tumors with *N-myc* amplification [230, 231]. Later, Kaghad et al reported monoallelic *TP73* expression in NB cell lines and pointed out the potential significance of this mechanism, particularly in tumors displaying 1p36 LOH, as deletion of the active allele may result in complete loss of p73 activity [159]. It is therefore very important to know the status of the allelic expression of *TP73* in normal and tumor tissues, since the frequency of 1p36 LOH varies significantly among human cancers, as has previously been outlined. Although most studies report biallelic expression of *TP73* in normal and malignant tissues, evidence still remains confusing.

Kovalev et al and Liu et al reported biallelic *TP73* expression in 4/8 and 5/6 neuroblastomas respectively [187, 190]. It was shown that NB cell lines displaying allelic loss of the gene still expressed p73 mRNA, providing further evidence for biallelic expression [186]. On the other hand, Ejeskär et al reported maternal or paternal loss of 1p in 2/5 and 3/5 NB tumors, respectively. Tumors with paternal loss of 1p did not express p73 mRNA, in contrast to the ones with maternal 1p-loss,

providing evidence that p73 is expressed from the paternal allele in tumors of advanced stage [191].

Evidence for *TP73* allelic expression in hematological malignancies have been perplexed. Kawano et al identified biallelic expression in human cell lines of AML, pre B-AML, cervical cancer and osteosarcoma, as well as in normal peripheral blood mononuclear cells [217]. On the contrary, Stirewalt et al reported monoallelic *TP73* expression in 6/10 *de novo* AML patients and one AML cell line [218], which had previously been analyzed by Kawano et al. In the same study, biallelic expression was detected in normal bone marrows [218]. Somewhat opposing results were obtained from another study, in which biallelic *TP73* expression was identified in 15/41 AML patients, whereas *TP73* was monoallelically expressed in 40/44 chronic myelogenous leukemia (CML) patients, 6/9 normal leukocytes, 2/8 granulocytes, 4/5 lympocytes and 2/4 $CD34^+$ progenitor cells [232].

TP73 is mostly biallelically expressed in ovarian cancers and normal ovaries, as has been shown in previous studies. Codegoni et al found biallelic expression in 5/6 ovarian tumors, 6/7 borderline ovarian tumors and in 1 normal ovary [226]. These results were later confirmed by Chen et al, who detected biallelic *TP73* expression in 22/24 ovarian carcinomas, 1/5 ovarian cancer cell line and 17/24 normal ovaries [228]. Interestingly, p73 expression was restored by treatment with the demethylating agent 5´-azacytidine (5´-aza) of three cell lines not expressing p73 protein and one tumor monoallelically expressing *TP73*, providing evidence for promoter methylation rather than imprinting as the epigenetic mechanism underlying p73 lack of expression, at least in ovarian tumors [228]. The same findings were reported by Kang et al in four p73 non-expressing gastric cancer cell lines. In the same study, monoallelic *TP73* expression was identified in normal gastric tissues, hamartomas and hyperplastic polyps, whereas 5/39 of gastric tumors overexpressing p73α were identified as biallelic expressors [207].

TP73 was also found to be biallelically expressed in 8/14 informative cases of invasive breast carcinomas [197], 7/8 informative hepatocellular carcinomas and in 22 of their

noncancerous counterparts [201], as well as in malignant and normal prostate tissues [215].

Loss of imprinting, i.e. transition from monoallelic expression in normal tissue to biallelic expression in cancer, was concurrently described by the same group in lung and renal carcinomas [199, 233]. Mai et al reported monoallelic *TP73* expression in normal samples but biallelic expression in matched tumors of the lung in five informative cases, pointing out the activation of the silent allele [199]. Monoallelic *TP73* expression was reported in normal renal tissues of 11/12 informative individuals but biallelic expression was shown in 7/11 of the matching renal tumors. Of particular note, biallelic expression of the allele not identified in normal tissues was found in 2/7 renal tumors, indicating allele switching. In the same study, one fetal thymic and two fetal pancreatic tissues monoallelically expressing *TP73* were analyzed and the expressed allele was found to be of maternal origin [233].

To our knowledge, the status of *TP73* allelic expression in bladder cancer has so far been investigated in two studies. Chi et al found biallelic expression in 12/23 informative bladder carcinomas, eight of which expressed high levels of p73 mRNA. On the contrary, none of the informative samples obtained from noncancer patients showed biallelic expression. Elevated p73 mRNA levels were also measured in 2/11 monoallelic *TP73* expressors, providing evidence for loss of imprinting in bladder malignancies. Interestingly, a neuroblastoma cell line previously reported to monoallelically express *TP73* was identified as a biallelic expressor [219]. Finally, Yokomizo et al reported biallelic *TP73* expression in all bladder tumors and matched normal tissues analyzed [234].

3.3. Regulation and function of the p53 family proteins

3.3.1. Regulation and function of the p53 protein isoforms

The p53 protein is a transcription factor that has been designated as "the guardian of the genome", as it is indispensable for the regulation of cell cycle control and safeguards cell stability by ensuring genome integrity. A plethora of cellular processes are mediated by p53 function: cell cycle arrest at G1/S checkpoint, apoptosis/programmed cell death, DNA repair and genome integrity control, response to stress conditions (hypoxia, DNA damage, depletion of ribonucleoside triphosphate pools, oncogene activation) and induction of senescence [235, 236].

Regulation of p53 occurs at both the transcriptional and translational levels; mechanisms regulating p53 protein conformation have also been described. Regulation of p53 is mainly carried out by mechanisms controlling p53 protein stability and/or degradation. The proteins Mdm2, $p14^{ARF}$ and E2F1 have been recognized as key modulators of p53 stability [237].

Mdm2 protein targets p53 protein for proteasomal degradation by ubiquitination. It also inhibits the transactivating ability of p53 by binding to its N-terminus, where the TAD is located. Given that the *Mdm2* gene is activated by p53, an autoregulatory feedback loop exists between p53 and Mdm2. Upon cellular stress signals, phosphorylation of p53 or Mdm2 has been observed to result in reduced p53-Mdm2 interaction [237].

$p14^{ARF}$ protein is encoded by the INK4a-ARF locus, which, like *TP53,* is frequently altered in human tumors. $p14^{ARF}$ prevents degradation of p53 by binding to Mdm2 in a region distinct from its p53-binding domain, so that p53-Mdm2 interaction is not perturbed. c-Abl protein binding to p53 has been described to have the same effect. $p14^{ARF}$ can also prevent p53 ubiquitination by Mdm2 and auto-ubiquitination of Mdm2 as well, by interfering with Mdm2's E3 ligase activity. In addition, $p14^{ARF}$ can lead Mdm2 from the nucleoplasm back into the nucleolus, leaving p53 in the nucleoplasm, where it can transactivate its target genes [237].

E2F1, a member of the E2F family of transcription factors, has been shown to activate apoptosis in cases of abnormal proliferative signals both dependently and independently of p53. Deregulated E2F1 expression results in the stabilization and activation of p53, which is at least partially mediated by E2F1 activating transcription of ARF. E2F1 deregulation in response to activated oncogenes such as Ras, Myc, v-Abl and E1A can also stabilize p53, not necessarily via ARF. Of note, E2F1 is also targeted for proteasome-dependent degradation by Mdm2, which reflects the complexity of the regulatory activities shared by p53 itself, Mdm2 and E2F1 [237].

p53 is activated by a variety of stress signals, such as DNA damage, oncogene activation, hypoxia, depletion of the ribonucleoside triphosphate pools, mitotic spindle damage and nitric oxide production [237]. Here the mechanisms of p53 activation by DNA damage and oncogene activation will be outlined.

DNA damage can result in p53 activation via upstream mediators, which include protein kinases, transcriptional activators with histone acetyltransferase (HAC) activity and inhibitors of transcription with histone deacetylase (HDAC) activity; also via other molecules, such as Sumo-1 protein and PML complex. DNA damage activates ATM and ATR kinases, along with Checkpoint kinases 1 and 2 (CHK1 and CHK2), which subsequently phosphorylate p53 and/or Mdm2. Phosphorylation of p53 and/or Mdm2 activates p53 through (i) abrogation of p53- Mdm2 interaction and prevention of p53 degradation, (ii) regulation of p53 transcriptional activity and (iii) promotion of p53 nuclear localization. On the other hand, oncogenes can activate p53 by modulating the transcription and/or function of the ARF gene. Oncogenes like E2F1, Ras, Myc and DMP1 can enhance *ARF* transcription, whereas Twist, Bmi-1 and TBX2 exert the opposite effect. Moreover, levels of p53 and its function can be modulated by post-transcriptional modifications, like SUMOylation [236].

Upon its activation, p53 protein can in turn activate or inhibit transcription of its downstream target genes by recruiting transcriptional activators or suppressors in their promoter regions. p53 can thus activate intracellular pathways which control cell cycle arrest, apoptosis, angiogenesis inhibition, suppression of metastasis and DNA repair [236].

p53 can induce cell cycle arrest at the G1/S checkpoint by activating p21, a known cyclin-dependent kinase 2 (CDK2) inhibitor. Cyclin-dependent kinases can phosphorylate many target molecules associated with cell cycle progression, so their inhibition results in cell cycle arrest [236].

p53 can induce apoptosis via the extrinsic and the intrinsic signaling pathways. The extrinsic pathway involves engagement of particular transmembrane receptors, namely Fas, DR5 and PERP. Transcription of the respective genes is induced by either γ-irradiation (for Fas) or DNA damage (for DR5 and PERP). Upon formation of the death-inducing-signaling complex (DISC), consisting mainly of Fas receptor molecules, the caspase cascade is activated beginning with caspase-3 and caspase-8, which in turn induce apoptosis. The intrinsic pathway is controlled by the Bcl-2 family of proteins, which comprises of both anti-apoptotic and pro-apoptotic members. *Bax*, *PUMA* and *NOXA* are pro-apoptotic genes transactivated by p53 in response to γ-irradiation and chemotherapeutic agents, DNA damage and possibly hypoxia, and X-ray irradiation, respectively. Bax, PUMA and NOXA proteins co-operate so that cytochrome c is released from the mitochondria. A protein complex called apoptosome is subsequently formed by APAF-1 and pro-caspase-9 molecules. APAF-1 is activated by p53 and its interaction with cytochrome c is essential for the apoptosome formation. Pro-caspase-9 is activated within the apoptosome and promotes activation of caspase-3, caspase-6 and caspase-7, again leading to apoptosis. Although these pathways are separately discussed, it should be noted that they are regarded by many as converging rather than diverging [238].

p53 can also transactivate genes that code for angiogenesis inhibitors, such as TSP-1, BAI-1, MASPIN and GD-AiF and genes involved in the suppression of metastasis, such as KAI-1 [236].

Last, p53 can transactivate genes coding for proteins responsible for DNA repair, e.g. p48 which takes part in the nucleotide excision repair (NER) mechanism and ribonucleotide reductase M2 B [236].

Bourdon et al reported that p53 isoforms have different subcellular localizations, suggesting that they may have diverse functions. Full-length p53, and p53β are

localized mainly in the nucleus and rarely in the cytoplasm, while p53γ appears shuttling between the nucleus and the cytoplasm. Δ133p53β is localized in the nucleus, whereas Δ133p53γ is detected only in the cytoplasm, indicating that the C-terminal residues can modify subcellular localization of these isoforms. Interestingly, distinct biochemical activities were identified for p53 isoforms, resulting in differential transcriptional activation of genes involved in apoptosis and cell cycle control.

In the same study, p53β was shown to preferentially bind to the *p21* and *Bax* rather than the *Mdm2* promoters. On the other hand, p53 preferentially bound to *Mdm2* and *p21* rather than *Bax* promoters. Upon forming protein complexes, p53 and p53β exhibit enhanced transcactivating activity on the *Bax* promoter in response to cellular stress, but not on the p21 promoter. Also, slightly increased p53-mediated apoptosis was observed after co-transfection of p53 with p53β. In striking contrast, co-transfection with Δ133p53 was shown to strongly inhibit p53-mediated apoptosis in a dose dependent manner. Also, Bourdon et al reported p53-independent transactivation by p53γ, which also enhanced p53 transcriptional activity on the *Bax* promoter [239]. These findings indicate that wild-type p53 activity may be modulated by p53 isoforms, which adds further complexity in the regulation of p53 activity in human normal and tumor tissues. Moreover, each p53 isoform may exert specific biologic functions, in co-operation with or independently of full-length p53.

Indeed, it was later shown by the same group that the internal *p53* promoter which generates the N-terminally truncated isoforms is directly transactivated by p53 in response to genotoxic stress, which in turns causes Δ113p53α protein induction. The Δ113p53α protein was found to inhibit p53-dependent apoptosis and G1 arrest, indicating that response to DNA damage may be modulated by p53 isoforms expression [240].

The Δ40p53 isoform, which lacks the N-terminal first transactivation domain, can form oligomers with p53 and thus impair its transactivating and growth-suppressive activities. In addition, this isoform does not interact with Mdm2 nor accumulates in response to DNA damage, suggesting that it is not involved in the cellular response to genotoxic stress [241]. Interestingly, it has been proposed that Δ40p53 expression is

regulated by alternative mechanisms of mRNA translation initiation employing an internal ribosome entry site (IRES) sequence, which allow expression under cytotoxic or endoplasmic reticulum (ER) stress or at the G2/M transition of the cell cycle. Δ40p53 expression was found to be increased after ER stress and Δ40p53 induced 14-3-3σ and G2 cell cycle arrest without affecting G1 progression, in contrast to wild-type p53, pointing out a new role of Δ40p53 in the p53 pathway [242].

3.3.2. Regulation and function of the p63 protein isoforms

Regulation of p63 isoforms has been described at the transcriptional, post-transcriptional and post-translational levels.

So far, few direct mechanisms that regulate transcription of TAp63 have been described. TAp63 is not induced by prominent p53 and TAp73 regulators, such as E2F1, as was shown by Waltermann et al in human keratinocytes. Additionally, it was observed that neither TAp63 nor ΔNp63 accummulated after imposing stress conditions (e.g. high density) to the cells [243].

Expression of TAp63α is up-regulated in response to DNA damage by doxorubicin, which increases affinity of the c-Jun transcription factor to the TAp63 promoter [244]. TAp63 can be induced by a variety of chemotherapeutic agents and its inhibition can result in chemoresistance. Gressner et al demonstrated that, in hepatoma cells treated with doxorubicin, cisplatin, bleomycin or mitroxantone, TAp63a directly transactivated the *CD95* gene, resulting in up-regulation of the CD95 death receptor. TAp63α also induced expression of genes involved in both the extrinsic and the intrinsic apoptotic pathways, such as *TNF-R1*, *TRAIL-R1*, *TRAIL-R2*, *BCL2L11*, *RAD9* and *APAF1*. Finally, it was shown that blocking of endogenous TAp63 by p63 siRNA protects cells against chemotherapeutic drugs due to inhibition of apoptosis and of caspase-3, caspase-8 and caspase-9 activation [245]. Of note, TAp63α was also found to up-regulate expression of oncogenes (*Maf*, *AML1*) and of genes involved in proliferation and differentiation, such as the Notch ligands *JAG1* and *JAG2* in a cDNA microarray analysis [246].

DNA damage is also known to down-regulate ΔNp63 while inducing TAp63. p53 stabilization and ΔNp63 down-regulation occur in parallel as p53 induces apoptosis in UV-treated human keratinocytes, suggesting opposing roles for p53 and ΔNp63 in response to DNA damage [247]. Both positive and negative regulation of ΔNp63 by p53 and by itself have been shown by different groups [243, 248] and evidence exists that ΔNp63 may be positively regulated by TAp63γ in a p53-independent manner [249].

Moreover, it has been reported that activation of the phosphatidylinositol 3-kinase (PI3-K) signaling pathway by epidermal growth factor and its receptor (EGFR) can promote ΔNp63α transcription in human keratinocytes and squamous carcinoma cells. A positive feedback loop may exist between p63 and EGFR, since p63 activates transcription of EGFR. Similarly, Notch signaling negatively regulates ΔNp63α expression, while ΔNp63α represses some Notch-induced genes [250].

Micro RNAs (mi-RNAs) have been identified as post-transcriptional regulators of p63 isoforms. The mi-RNA miR-21 targets TAp63 in glioblastoma cells, thus promoting tumor growth, whereas ΔNp63β is inhibited by miR-92 in myeloid cells. During skin development, miR-203 directly targets and represses ΔNp63 isoforms [250].

Post-translational regulation of p63 takes place by phosphorylation and ubiquitination. UV exposure and cisplatin treatment promote increased ΔNp63α phosphorylation and proteasome-dependent protein degradation. p63α isoforms can also be regulated, although rarely, by SUMO-1, a small ubiquitin-like modifier [250, 251].

Apart from their roles in apoptosis, p63 isoforms have opposing functions in cell cycle control, depending on the cell type. In erythroleukemia cells, TAp63γ accumulates in response to genotoxic stress and can cause cell cycle arrest by inducing p21 expression. On the contrary, exogenous ΔNp63α binds to the *p21* promoter and inhibits transcription [251]. In human and mouse primary keratinocytes, down-regulation of ΔNp63α results in increased p21 expression and decreased cyclin and cyclin-dependent kinases expression, thus reducing proliferation and promoting cell cycle arrest [250, 251].

When cloning the *TP63* gene, Yang et al reported that the TAp63γ isoform showed stronger transcriptional activation of a reporter gene under the control of a p53-responsive element when compared to TAp63α. Moreover, higher apoptotic levels were reported in p53-mutant expressing cells transfected with TAp63γ, whereas apoptosis was virtually absent in cells expressing high levels of ΔNp63α or TAp63α. It was also clearly shown that ΔNp63α and ΔNp63γ could inhibit transactivation activity of p53 in a dose-dependent manner. Interestingly, slightly low apoptotic levels were measured in cells expressing ΔNp63γ and slightly increased transactivation of the reporter gene was noted in cells co-transfected with TAp63γ and ΔNp63γ at a ratio of 5:1. Nevertheless, higher levels of ΔNp63γ inhibited transactivation by TAp63γ. Also, ΔNp63α was found to strongly suppress both TAp63γ and p53. Finally, it was shown that ΔNp63γ (and TAp63α) can specifically interact with p53 target sites, a finding which could explain the suppressive function of the ΔN isoforms over the full-length ones and over p53 [158]. It was later demonstrated that ΔNp63α binds to the *p21* and *14-3-3σ* promoters and represses transcription both *in vitro* and in primary human epidermal keratinocytes (HEKs). In accordance with Yang et al, Westfall et al also found that the predominant isoform expressed in primary HEKs is ΔNp63α. It was also shown that its expression, along with its binding to p53 target sites, decreased with keratinocyte differentiation, pointing out an important role of ΔNp63α in basal keratinocyte proliferation [252].

3.3.3. Regulation and function of the p73 protein isoforms

So far, regulation of p73 proteins has been described at the transcriptional and post-translational levels, mainly by accumulation of p73 protein, phosphorylation and rarely by ubiquitin-like modifications [167, 250].

p73 activity is regulated by many of the same mechanisms controlling p53 but not p63. Waltermann et al showed that TAp73 expression was induced by E2F1, a known key modulator of p53 stability, but inhibited by TGFβ in human keratinocytes. Also, exogenous expression of p53 induced transcription from the internal *TP73* promoter, resulting in ΔNp73 mRNA and protein accumulation. Although ΔNp73 was enhanced,

ΔNp63 was reduced, suggesting differential regulation of p63 and p73 by their homologue [243]. Interestingly, inactivation of p53 may up-regulate expression of TAp73 in breast cancer cells through E2F1-mediated transcriptional regulation. This suggests that an intrinsic "complementary" mechanism may exist, which is potentially activated in response to p53 loss [253].

The P1 promoter if the *TP73* gene has 3 E2F1-binding sites, through which E2F1 directly activates TAp73 expression and apoptosis in the absence of p53 [254]. Activation of E2F1 by the c-Myc oncogene or the viral oncoprotein E1A also results in TAp73α and TAp73β induction to promote apoptosis [184]. There is also evidence that TAp73 activation by E2F1 can mediate DNA damage response in p53-mutant cells. Moreover, c-myc has been described to increase TAp73 protein levels, as well as to regulate p73-dependent transcription [250].

TAp73 has been shown to accumulate and induce apoptosis in mouse embryo fibroblasts (MEFs) and human colon cancer cells treated with cisplatin. Interestingly, accumulation of p73 required both c-Abl tyrosine kinase and mismatch-repair protein MLH1, suggesting that p73 and c-Abl participate in a mismatch repair-dependent apoptotic pathway [255]. Of note, p73 is not induced by ultraviolet (UV) light, actinomycin D, ionizing radiation (IR) or methylmethane sulfonate in colon cancer and neuroblastoma cell lines, indicating that p73-mediated response to cytotoxic stress is cell type-specific and dependent on the cause of DNA damage [159, 167].

c-Abl protein kinase can stabilize TAp73 following genotoxic stress by phosphorylation of its Tyr99 residue, thus facilitating TAp73 pro-apoptotic function. Most strikingly, c-Abl-mediated stabilization of ΔNp73 has also been reported in cisplatin-treated cancer cells, yet the significance of this has not yet been investigated [256]. On the contrary, p73 phosphorylation on its Thr 86 residue by certain cyclin/CDK complexes involved in S-phase and G2/M progression was found to inhibit p73 activity [257].

p73 protein stability is also controlled by proteasomal-dependent degradation, yet association of p73 with Mdm2 does not induce ubiquitination and degradation but rather contributes to p73 protein stability. p73 can induce Mdm2 and subsequent binding of p73 to Mdm2 can reduce its transcriptional activity by inhibiting p73

interactions with transcriptional co-activators [184, 250, 258]. The p73-Mdm2 interaction might be of importance, as Mdm2 sequestering can promote apoptosis by increasing p53 levels. Degradation of p73 is apparently regulated through SUMOylation modifications by SUMO-1 [184].

TAp73 isoforms exert their pro-apoptotic and growth-inhibiting functions by activating the transcription of several apoptotic effectors, which are also p53 and/or p63 targets, like *Bax*, *NOXA*, *PUMA*, *CD95* and *p21* [159, 184]. Shortly after the discovery of *TP63* and *TP73*, it was shown in MEFs that p53 requires both TAp63 and TAp73 to promote cell death following DNA damage by selectively activating apoptotic promoters like the aforementioned ones [259]. Interestingly, p73 and in particular its TAp73 isoforms, have recently been implicated in mitosis and maintenance of genomic stability. It has been shown in p73-null MEFs that cells become aneuploid or polyploid, independently of p53 status. Also, mitotic defects such as lagging chromosomes and aberrant mitotic spindle formation have been described in oocytes and fibroblasts from TAp73 selective knockout mice [257].

On the other hand, ΔNp73 isoforms have been shown to suppress apoptosis and enhance oncogenic signaling. Petrenko et al showed that ΔNp73 can promote cell proliferation and colony formation in MEFs, while at the same time inhibiting p53 mediated apoptosis. ΔNp73 is also able to co-operate with Ras and transform MEFs *in vitro*, as well as to promote tumor formation *in vivo* in nude mice. Importantly, it was shown that p53 is targeted from ΔNp73, since deletion of p53 or ARF disrupted the growth-promoting effect of ΔNp73 [260]. Recently, it was reported that ΔNp73 can reduce TAp73-mediated activation of a *p21* promoter reporter gene in a dose-dependent manner. ΔNp73 also induced transcription from a PAI-1 promoter reporter gene, even when specific p53-binding elements (p53BEs) were absent, suggesting that ΔNp73 might exert its transactivational function by using regulatory elements other than p53 and/or p63. In addition, it was shown both in hepatocarcinoma and embryonic kidney cells that ΔNp73 can enhance transcription of TGF-β, a gene involved in invasion and metastasis [261]. The dominant negative properties of ΔNp73 isoforms over TAp73 and p53 were demonstrated by Grob et al. ΔNp73 was found to block the ability of TAp73 and p53 to bind to the *p21* promoter in a dose-dependent

manner. Moreover, ΔNp73 significantly reduced TAp73- or p53-mediated apoptosis after co-transfection in Saos-2 cells. Most importantly, it was shown that p53 inhibition is achieved through competition for the binding to the specific p53BEs, whereas TAp73 is inhibited through hetero-oligomer formation [262].

3.4. Role of the *TP53* family members in development

p53-null mice develop multiple types of tumors by their first 6 months of age, yet they lack any observable developmental defects [263]. On the contrary, p63- and p73-null mice lack spontaneous tumors but are born with severe developmental defects, if not dead.

p63-deficient mice fail to maintain the apical ectodermal ridge and are born with truncated limbs and a striking absence of all squamous epithelia and their derivatives (mammary, lachrymal and salivary glands) [264].

In normal human tissues, p63 protein is expressed in basal cells of the epithelium in foreskin, cervix, vagina, urinary bladder and prostate. Yang et al first showed that the ΔNp63α is predominantly expressed in human keratinocytes, in which TAp63 is not detected [158]. Indeed, a greater role in developmental processes has been documented for ΔNp63 rather than TAp63 in various studies.

In mice, the ectoderm is known to commit and differentiate into various epithelial lineages at embryonic day 12.5 and ΔNp63 is expressed as early as embryonic day 11.5. ΔNp63 can induce genes involved in first epidermal stratification, whereas it represses mesodermal-specific genes [265]. Laurikkala et al detected the ΔNp63α isoform during the early stages of development, when teeth and hair are formed. Genes involved in cell differentiation and development, like *Bmp7* and *Notch1* were recognized as potential transcriptional targets of ΔNp63α [266].

ΔNp63 has a clearly distinct role in epidermal morphogenesis than TAp63, as it regulates expression of basal layer markers of the epidermis (like K14), while TAp63 transactivates genes characteristic of the upper layer (K1, K10, loricrin) [267].

Pignon et al showed in mice that the basal, intermediate and umbrella cells of the adult urothelium, as well as all prostate epithelial lineages and the adult colorectal epithelium originate from ΔNp63-expressing cells [268]. Also, ΔNp63 has been identified to play an anti-apoptotic role in normal bladder development by Cheng et al, who also detected ΔNp63β and ΔNp63γ, apart from ΔNp63α, in mouse bladders. Interestingly, it was noted that the bladder epithelium in p63-null mice remained a single layer, whereas in wild-type animals it was shown to differentiate into stratified transitional urothelium. The p63-deficient bladders also contained little or no smooth muscle in the ventral wall and a thin layer in the dorsal wall. In addition, the lamina propria was either absent or greatly reduced and the detrusor muscle was disorganized and non-stratified. The absence of the abdominal and ventral bladder walls is identical to bladder exstrophy, a congenital anomaly affecting human neonates. Of note, significantly increased apoptotic levels, along with *p53*, *Bax* and *Apaf-1* activation, were observed in bladders of p63-null mice, whereas ΔNp63β and ΔNp63γ were found to negatively regulate *Bax* and *Apaf-1* in bladder cells [269].

p73-null mice are born with severe developmental defects, such as hydrocephalus and hippocampal dysgenesis. They also present defects of pheromone detection which result in lack of sexual interest in mature females. In addition to the neurological defects, p73-null mice suffer from generalized pan-mucositis and chronic infections characterized by massive neutrophil infiltration [270].

ΔNp73 isoforms have been identified as key regulators of neuronal cell maintenance and survival. Pozniak et al detected ΔNp73α but not TAp73 in the developing cortex of mice as early as embryonic day 19. ΔNp73α or ΔNp73β isoforms were shown to exert an anti-apoptotic function in cortical neurons exposed to either a DNA damaging agent or a PI3-K inhibitor. Loss of ΔNp63 isoforms resulted in gradual loss of cortical neurons after birth, a decrease in facial motor neuron number and in severe defects of the olfactory bulb. An anti-apoptotic role for ΔNp73 in sympathetic neurons had already been demonstrated by the same groups [272], who proposed that the N-terminally truncated p73 isoforms are essential not only for survival of central and peripheral nervous system neurons, but also for the long-term maintenance of at least some adult neurons [271].

Interestingly, TAp73 knockout mice, apart from hippocambal dysgenesis and spontaneous tumors, present infertility. Tomasini et al observed that the oocytes of TAp73-deficient mice could not progress to the fallopian tubes and that these animals produced fewer gametes than the wild-type ones. In addition, genomic instability was noted in TAp73-null mice, with aneuploidy occurring more frequently in TAp73-deficient cells than in p53-deficient or wild-type cells [273].

p73 isoforms have also been implicated in nephrogenesis. TAp73 is predominantly expressed in the differentiation domain of the renal cortex, where it induces developmentally regulated genes like aquaporin-2 (*AQP-2*) and bradykinin B2 (*B2R*) independently of p53. On the other hand, ΔNp73 isoforms are expressed early in development and in proliferating precursors in the nephrogenic zone, promoting cell survival. As maturation proceeds, renal epithelial cells accumulate TAp73 and down-regulate ΔNp73, indicating that ΔNp73 is required for maintenance of the undifferentiated state of the nephrogenic zone [274].

3.5. Expression of the *TP53* family genes in human normal and cancerous tissues and cell lines

3.5.1. Expression of the *TP53* gene

Wild-type p53 is a 393 amino-acid phosphoprotein which, under normal conditions, is kept at low levels within the cell due to its relatively short half-life of approximately 20 min [275].

A rapid increase in the levels of p53 is observed under conditions of (i) DNA damage caused by chemical agents, UV or γ-irradiation, (ii) hypoxia, (iii) ribonucleoside pool depletion etc. In such cases, the cell either lengthens the p53 protein half-life or enhances the rate of translational initiation of p53 mRNA. Frequently, this increase in the levels of p53 may be proportional to the extent of damage and/or stress imposed on the cell [275].

Cancer cells of several types harbor *TP53* mutations, which affect virtually every domain of the protein. Missense mutations within the DNA-binding domain coding sequence are the most frequent and result in a single amino-acid substitution, while p53 is stably produced by the tumor cell. Otherwise, loss of p53 protein expression is caused by frameshift or nonsense mutations [276].

Aberrant p53 expression has been found in a variety of human malignancies.

TP53 is mutated in the vast majority of ovarian carcinomas [171]. Mutations and protein overexpression are more prevalent in serous primary ovarian cancers than in endometrial, mucinous and clear-cell tumors [277].

In breast carcinomas, wild-type p53 protein has been detected in 33% of the tumors [222] and mutant p53 in 38% of the tumors [278]. Interestingly, p53 expression correlated with p73 and ΔNp73 expression, respectively.

Inactivation of p53 has also been correlated with p73 up-regulation in hepatocellular carcinomas (HCCs) [224]. A recent meta-analysis of 37 studies on the prognostic significance of p53 alterations in HCCs revealed that mutations and protein overexpression significantly associate with reduced overall and recurrence free survival [279].

p53 has been detected in 72% [278] and 59% [280] of colorectal adenocarcinomas. In the first study, Domínguez et al used a monoclonal antibody detecting 89% of *TP53* point mutations [278].

Abnormal p53 expression has been detected in 76.4% of esophageal squamous cell carcinomas [204]. Protein expression is also frequent in precursor lesions, such as chronic esophagitis and occur in up to 100% of squamous carcinomas [281].

In prostate cancer, p53 expression is strongly associated with the presence of exon 5-8 mutations. p53 protein is more frequently detected in late-stage disease including metastatic cancer and in hormone-refractory tumors [282].

p53 protein has been detected in 52% and 65% of non-small cell lung cancers (NSCLCs). Protein overexpression was found in 52% of 253 NSCLC patients undergoing complete tumor resection and was significantly associated with poor prognosis [283].

It has previously been reported that p53 isoforms follow a tissue-specific pattern of expression in normal human tissues, suggesting that the alternative promoter and exon splicing are regulated. The p53β and p53γ isoforms are expressed in most normal tissues but are not detected in lung, fetal brain and liver and spinal cord. Additionally, p53β is not detected in brain, prostate and muscle and p53γ is not detected in spleen and testis. The Δ133p53 variant is not detected in uterus, prostate, skeletal muscle and breast. The Δ133p53γ variant is not detected in brain, lung, heart, fetal liver, salivary gland, breast and intestine. The Δ133p53β variant is detected only in colon, bone marrow, fetal brain and intestine [239].

Several studies have reported abnormal expression of p53 isoforms in different types of human cancers.

Hofstetter et al analyzed p53 isoform expression in 245 primary ovarian carcinomas and reported that p53β expression was associated with serous and poorly differentiated tumors. They also reported frequent splice-site mutations leading to the expression of a tumor-specific splice variant, named p53δ. [284].

Bourdon et al recently reported the analysis of p53β and p53γ mRNA expression in 127 primary breast tumors. They identified that p53β is associated with p53γ and with estrogen receptor (ER) expression. p53γ was also associated with *TP53* gene mutation [285].

Song et al analyzed p53 isoform expression in 41 renal cell carcinomas (RCCs) matched to normal adjacent tissues. They detected p53, p53β, p53γ, Δ133p53α and Δ133p53γ in normal and cancerous samples, whereas Δ133p53γ was detected only in tumor tissues. p53 expression was associated with stage and grade. They also found significantly higher p53β mRNA levels in tumor than in normal tissues [286].

The p53β isoform was also most frequently detected in 21 head and neck squamous cell carcinomas (HNSCCs), 14 matched normal tissues and 8 buccal mucosa samples.

p53γ, Δ133p53α and Δ133p53γ were also detected. Nevertheless, expression of p53 isoforms did not correlate with the clinicopathological parameters [287].

Avery-Kiejda et al analyzed p53 isoform expression in 16 melanoma cell lines. p53β and Δ40p53 mRNA and protein were detected in the majority of cell lines but not in normal fibroblasts or melanocytes. Absent p53γ and Δ133p53γ were also reported in melanoma cells. Interestingly, it was shown that p53β can transactivate *p21* and *PUMA* promoters, whereas Δ40p53 inhibits the basal transcriptional activity of p53 on both promoters. In p53-negative melanoma cells, neither p53β nor Δ40p53 had the same effect on these promoters, suggesting that those isoforms have no transcriptional activity of their own [288].

In the human bladder, p53 protein appears to follow a distinct expression pattern in normal and tumor tissues. Karni-Schmidt et al demonstrated that, although p53 is undetectable in normal urothelium, its expression increases with disease stage, from 6.5% in pTa tumors, to 21.4% in pT1 tumors and 43.6% in ≥ pT2 tumors [289]. In accordance with these results, Compérat et al identified lower p53 protein levels in papillary urothelial neoplasms of low malignant potential (PUNLMPs) than in high-grade muscle-invasive lesions [290]. Aberrant p53 expression has been found in 56%, 39%, 18.9% and 41.6% bladder cancer patients in four independent studies [106, 109, 291, 292]. In these studies, p53 expression has been associated with higher risk for progression and death [106, 291], independently of stage, grade and lymph node status [291].

3.5.2. Expression of the *TP63* gene

In normal human tissues, the p63 protein has been detected in the basal cells of the epithelium in foreskin, cervix, vaginal epithelium, urothelium and prostate [158].

Di Como et al studied p63 protein expression in a broad spectrum of human normal and tumor tissues. Strong p63 expression was identified in epithelial cells of the esophagus, tonsil, exocervix, prostate, larynx, lung, epidermis, hair follicles, sweat glands and urinary bladder. They also detected p63 protein in 3/4 transitional cell

carcinomas (TCCs) of the bladder, 4/4 head and neck squamous cell carcinomas (HNSCCs), 3/4 squamous cell carcinomas (SCCs) of the cervix, 2/4 SCCs of the lung, 2/4 salivary gland carcinomas, 4/4 parotid tumors and 13/13 thymomas, 6/8 teratomas of the testis and 2/4 teratomas of the ovary [293].

p63 overexpression is a frequent event in SCCs [294]. Hibi et al reported p63 mRNA up-regulation in 10/14 SCCs of the lung and in 6 HNSCC cell lines, in some cases as high as 5-fold. They also detected positive p63 staining in 10/23 lung cancers, 9/9 primary HNSCCs and in all HNSCC cell lines [177]. Intense p63 protein staining has been found in 50/51 esophageal tumors examined by Hu et al. Interestingly, 90.9% adjacent dysplastic lesions and 96.0% of normal adjacent epithelia showed strong p63 staining as well [295].

The N-terminally truncated ΔNp63 isoforms, and particularly ΔNp63α, are predominantly expressed, at the mRNA and protein levels, in SCCs of the esophagus [295], head and neck [296-298], lung [299] and cervix [300], often in their normal counterparts as well [295, 296, 298]. On the contrary, TAp63 isoforms are rarely detected in normal and malignant squamous epithelia [295, 297, 298]. In a study by Rocco et al, ΔNp63 mRNA and protein were both up-regulated by 3.5-8 times in HNSCC cells compared to human keratinocytes and ΔNp63 mRNA was found over a 100-fold more abundant than TAp63 mRNA in all cells tested [297]. Also, significantly higher ΔNp63 transcriptional levels have been identified in HNSCCs compared to normal samples [298]. Massion et al detected ΔNp63α protein in normal bronchus and SCCs of the lung, but not in normal lung and adenocarcinomas [299].

The *TP63* gene is also implicated in hematological malignancies and p63α was mostly expressed in 72/74 chronic myelogenous leukemia (CML) patients [178]. In contrast to SCCs, TAp63 and not ΔNp63 isoforms are detected in lymphomas [301, 302]. Interestingly, higher TAp63 mRNA levels were detected in 44/45 follicular lymphomas compared to normal lymphocytes and protein expression was significantly correlated with transcriptional up-regulation [301]. In addition, Zamò et al detected TAp63 protein expression in 30-80% of neoplastic cells in 13 primary mediastinal large B-cell lymphoma patients. Interestingly, TAp63γ mRNA was overexpressed in primary

mediastinal large B-cell lymphoma up to 115.21-fold compared to germinal center B cells and up to 5.16-fold compared to diffuse large B-cell lymphoma [302].

Increased TAp63 protein levels were also measured in intestinal metaplasia and atrophic gastritis, which are considered as precursors to gastric adenocarcinoma [208].

Loss of p63 expression in invasive breast carcinomas has been described in three independent studies [303-305]. p63 protein was detected almost exclusively in the myoepithelial cells of the normal breast and in benign lesions and rarely in ductal hyperplasia, ductal carcinoma *in situ* and invasive papillary carcinoma [303-306].

Prostate adenocarcinomas also appear to be devoid of p63 protein. Parsons et al noted weak p63 intensity in less than 1% of the malignant cells in 21/233 prostate adenocarcinomas [307].

Park et al studied 47 urothelial carcinomas, 6 of which were matched to normal tissue. They found that TAp63 mRNA was down-regulated in 53.2% and that ΔNp63 was up-regulated in 63.8% of the samples. TAp63 down-regulation and ΔNp63 up-regulation was tumor-specific in 2 and 3 tissue pairs, respectively [307]. In E-cadherin-positive urothelial carcinoma cells, ΔNp63 transcripts were greatly enhanced over TAp63 transcripts [308]. Similarly, TAp63 was down-regulated and ΔNp63 was up-regulated in all 49 tumors analyzed by Compérat et al [309]. Most authors agree that ΔNp63 is predominantly expressed in the malignant and is undetectable in normal bladder, whereas TAp63 is absent from tumors and retained in the normal urothelium [289, 307, 308, 310]. Urist et al identified multiple TA- and ΔNp63 isoforms expression in non-muscle-invasive transitional carcinoma cells, yet suggested that ΔNp63 is the major isoform expressed in invasive transitional and squamous carcinomas of the bladder [310]. In striking contrast, Koga et al reported that ΔNp63 mRNA was abundant in normal samples, whereas TAp63 was undetectable, and that tumor tissues expressed variable levels of ΔNp63 mRNA [311]. A gradual decrease in p63 protein expression with tumor stage has been reported in five studies [289, 290, 309-311]. p63 protein was detected in 96.8-100% of the pTa, 80-94% of the pT1 and 73% of the ≥ pT2 tumors examined [289, 308]. Koga et al identified heterogeneous p63 immunoreactivity in 75 carcinomas under study and denoted its distribution as

"chaotic", particularly in invasive lesions [312]. It was also reported that metastatic lymph nodes and *in situ* carcinomas exhibited p63 expression identical to that of the primary lesions [311].

3.5.3. Expression of the *TP73* gene

In normal human tissues, p73α and p73β have been detected, albeit in low levels, in brain, kidney, placenta, colon, heart, liver, spleen and skeletal muscle [159]. Puig et al detected strong p73α protein expression in normal esophagus, tonsil, testis, larynx and bladder [313].

In neuroblastoma (NB) tumors, p73α and p73β transcripts have been detected in 34% and 75% of the tumors [185, 192] and are frequently co-expressed [192]. In normal tissues (thyroid, kidney, uterus, neutrophils etc) examined along with the tumors, very low p73 mRNA levels have been detected [187]. Also, some groups have reported down-regulated p73 transcripts in NB tumors and cell lines as well [187, 188]. On the contrary, Yang et al detected aberrant p73 mRNA expression in 25% of primary mass-screening tumors and in 71.4% of clinically found tumors [186]. TAp73 mRNA expression has rarely been reported in NB and in one study 10% of the tumors were found to express only ΔNp73 but not TAp73 transcripts [314]. Interestingly, Kovalev et al reported 8-80-fold increased p73 transcripts in one ganglioblastoma, one sarcoma and one desmoplastic small round cell tumor. In the NB cell lines, p73 was up-regulated 8-90-fold as compared to the mean normal tissue level [187]. p73α protein has been detected in 83% of the tumors examined, whereas ΔNp73α in particular was detected more frequently in undifferentiated NB tumors [192].

High p73 protein levels have also been found in ependymomas [315]. In another study, Wager et al analyzed 51 gliomas and 3 normal samples and found up-regulated TAp73, ex2p73 and ex2/3p73 transcripts [316].

In hematological malignancies, p73 appears to be differentially expressed depending on the lesion type of the differentiation status of the cells. p63 α-ζ isoforms have been detected in leukemic blasts and acute myeloid leukemias (AMLs), yet not in normal or

mature myeloid leukemic cells [317, 318]. Kowano et al studied 39 samples from patients with leukemia, lymphoma or myeloma and a substantial number of cell lines, including pre-B/B-acute lymphoblastic leukemias (ALL), T-ALL/lymphoblastic lymphomas (LBL), B-non-Hodgkin's lymphomas (B-NHL), AMLs, T-NHLs and multiple myelomas. Whereas p73 mRNA was detected in all non-hematopoietic cancer cell lines simultaneously analyzed (NB, breast, colon, prostate etc), 32% of ALL/B-NHL cell lines expressed low or undetected p73 transcripts [217]. Peters et al detected p73 mRNA in 30/31 CML and in 32/35 AML patients, 9/13 leukemia cell lines and in all normal peripheral blood cells. CML patients were found to express the highest p73 levels. They also reported strong p73 protein expression in 4/9 leukemia cell lines [231]. By other groups, high p73 mRNA levels have been measured in AML patients [218, 318]. ΔNp73 mRNA and protein were detected in 27/28 AML patients, yet expressed only in a subset of 13/41 of acute promyelocytic leukemia (APL) patients [318]. Higher TAp73 and ΔNp73 transcripts were detected by Leupin et al in 51 B-cell chronic lymphocytic leukemia patients as compared to the normal controls [319].

While p73 expression is nearly absent from normal ovaries [227], high mRNA and protein levels have been measured in invasive and borderline ovarian tumors and cell lines [211, 227, 228]. A marked de-regulation of p73 isoforms expression has been reported by two groups. Zaika et al reported 2-36-fold ΔNp73 up-regulation and 3-42-fold TAp73 up-regulation in ovarian tumors as compared to normal tissues [320]. Similarly, Concin et al reported TAp73 up-regulation in 1/3 of ovarian carcinomas and ΔN´p73 tumor-specific up-regulation in almost all samples (95%). Very low levels of ex2p72 were measured and ex2/3p73 was down-regulated in tumor tissues as compared to normal ones [321]. Hofsetter et al recently confirmed that ΔN´p73 is the most frequently expressed N-terminally truncated variant in ovarian cancer [284].

Deregulation of p73 expression has also been observed in other gynecological malignancies, such as cervical, endometrial and vulval cancer. Significant overexpression of both TAp73 and ΔNp73 mRNA in cervical SCCS as compared to normal tissues has been reported [322]. In accordance, Zaika et al reported 25-78-fold ΔNp73 up-regulation and 7-155-fold TAp73 up-regulation in cervical carcinomas as compared to normal tissues. In endometrial tumors, ΔNp73 was up-regulated by 6-

150-fold, TAp73 was up-regulated by 6.5-32-fold and ex2p73 was up-regulated by 2.4-20-fold as compared to normal tissues [320]. The ex2p73 isoform mRNA is also up-regulated in vulval tumors, in which TAp73 is rarely overexpressed [212, 320].

Whereas normal breast tissues express low levels of p73 and predominantly p73α [197], p73 mRNA up-regulation is a frequent event in breast carcinomas [197, 222, 223]. A marked increase of 5-25-fold and 13-73-fold in p73 mRNA levels was observed in 38% of the tumors and 5/7 cell lines analyzed by Zaika et al [197]. Domínguez et al reported frequent co-expression of TAp73, ΔNp73, ex2p73 and ex2/3p73 isoforms in 60 breast tumors and identified a good correlation between TAp73 and ΔNp73 mRNA and protein [278]. Two-fold and 3-fold higher p73 mRNA levels were also measured in breast carcinomas with no inflammatory symptoms (NBC) and in inflammatory breast carcinomas (IBC) respectively, when compared to normal tissues [223].

The TAp73 isoform is predominantly expressed in normal hepatic tissue [323], whereas ΔNp73 mRNA up-regulation has been found in hepatocellular carcinomas and cell lines [324, 325]. In a later study, Stiewe et al reported down-regulation of the ΔN promoter and increased transcriptional levels of ex2p73 (up to 31.5-fold), ex2/3p73 (up to 7.75-fold) and ΔN´p73 (up to 16.3-fold) in hepatocellular carcinomas and cell lines [323].

Higher p73 mRNA levels have been detected in colorectal tumors compared to their normal counterparts [209] and p73 protein expression was found to increase from 19% of the normal intestinal mucosa to 67% of primary colorectal carcinomas and 95% of metastatic tumors [280]. In colorectal tumors, TAp73, ΔNp73, ex2p73 and ex2/3p73 mRNAs are expressed [278, 326], whereas TAp73 and ΔNp73 proteins were detected in good correlation with the respective transcripts [278].

In lung cancers, two early studies reported higher p73 mRNA levels in a total of 107 primary tumors compared to normal samples [199, 327]. On the contrary, Liu et al later reported loss of p73 transcripts and protein in 6 NSCLC cell lines, which was linked to aberrant methylation [328].

Significantly higher p73 mRNA levels compared to normal tissues have been detected in SCCs of the esophagus [204] and p73 protein expression was detected in 68.4% of

SCCs of the head and neck, particularly in p53-negative tumors [294]. On the contrary, reduced p73 transcripts were measured in 5/17 SCCs of the head and neck by Faridoni-Laurens et al [210].

Both p73 mRNA and protein, particularly p73α, have been detected in gastric adenocarcinomas [207, 208]. Kang et al reported tumor-specific p73 overexpression in 14/16 gastric tumors matched to normal tissues, yet p73 mRNA was undetected in 4/5 gastric carcinoma cell lines [207].

Early data on p73 expression in melanomas were contradictory. Schittek et al reported low or absent p73 mRNA levels in benign lesions, primary melanomas and lymph node metastases, yet detected various levels of p73 in all 9 cell lines [213]. On the contrary, Kroiss et al reported significant p73 mRNA expression in melanoma cell lines, benign nevi, primary and metastatic tumors [214].

Early data did not implicate p73 in prostate tumorigenesis. Yokomizo et al detected no differences in p73 mRNA levels between prostate tumors and normal tissues [215], whereas Takahashi et al reported low p73 mRNA levels, particularly of the p73α isoform, in prostate tumors [216]. A later study though, showed significantly increased expression of TAp73 and ΔNp73 in 24/33 and 20/33 of the prostate tumors respectively, as well in benign prostate hyperplasias and prostatic carcinoma cell lines [329].

In bladder tumors, p73 is frequently up-regulated. Yokomizo et al reported p73 overexpression in 22/23 primary muscle-invasive transitional cell carcinomas of the bladder. They also detected p73α and p73β transcripts in both normal and malignant bladder tissues, at an expression ratio of 1.6:1 [234]. Significantly elevated p73 transcripts were also measured in 18/45 of primary bladder cancer analyzed by Chi et al, although only p73α were detected [219]. Puig et al studied p73 expression in 154 transitional cell carcinomas of the bladder, 8 transitional cell carcinoma-derived cell lines and one squamous cell carcinoma-derived cell line. p73α protein expression was detected in 57% of non-muscle-invasive tumors but only in 21% of the muscle-invasive ones. ΔNp73 mRNA was detected in all cell lines, but TAp73 transcripts were not detected in 4/7 muscle-invasive cell lines and in one transitional cell carcinoma-derived

cell line. A good correlation was observed between TAp73 mRNA and protein levels in cell lines which retained TAp73 expression. Finally, the squamous cell carcinoma-derived cell line expressed only the ΔNp73 isoform [313].

4. STUDY OBJECTIVE

Urinary bladder cancer is the 11th most common malignancy worldwide, with significant share in cancer-related morbidity and mortality [7, 86].

Studies on the molecular alterations taking place in bladder cancer have focused on the identification of specific genetic factors that contribute to malignant transformation and to disease progression, which can be either compromising for patients' quality of life or even be lethal. In the majority of studies, mutations in oncogenes and tumor suppressors is analyzed, along with their expression in bladder tumors and cell lines. Over the years, it has become evident that urothelial tumors present with two phenotypic variants which arise via distinct molecular pathways [86].

p53 is the most extensively studied tumor suppressor gene and 50% of human cancers have either lost p53 expression or express mutant p53 [157]. p63 and p73 genes are p53 homologues, yet exhibit notable differences with wild-type p53 in terms of their genomic organization and function [164]. Intensive studies have shown that p63 and p73 produce two major types of isoforms with tumor-suppressor and oncogenic properties [164].

Studies on the role of p53 family genes in bladder cancer have so far produced interesting results. In the current study, the transcriptional levels of the p53 family genes were analyzed in a set of 30 human urothelial tumors, matched with their normal adjacent tissue samples. Moreover, the transcriptional levels of the two major types of p63 and p73 isoforms were analyzed.

To the best of our knowledge, this is the first study to simultaneously analyze the transcriptional levels of all p53 family genes by quantitative Real-Time Polymerase Chain Reaction in bladder tumors and their non-malignant counterparts. Previous studies have analyzed p53 or p63 isoforms (TAp63 and ΔNp63) mRNA levels. Yet, no study to date but ours has analyzed TAp73 and ΔNp73 mRNA levels in solid human bladder tumors. An additional advantage of our study is that every tumor sample is

paired to a normal tissue specimen obtained from the same individual, which will make our results more reliable.

The objective of the current study is to produce an expression profile of the *TP53* family genes and the *TP63* and *TP73* isoforms in urothelial tumors, to identify possible associations with patients' clinicopathological parameters and to provide evidence of potential interactions between these genes in normal and malignant urothelium. With our results, we hope to contribute to deciphering the role of the p53 family genes in the development and progression of urothelial cancer.

5. MATERIALS AND METHODS

5.1. Methods

5.1.1. Sample collection

Thirty transitional cell carcinomas (TCCs) were collected from patients diagnosed with urinary bladder cancer (UBC) who underwent surgical resection of bladder tumor at the Department of Urology, Asklipeiion Hospital, Voula, Athens, Greece. Each tumor sample was matched to its non-malignant counterpart, which was obtained from normal adjacent bladder tissue using cold biopsy techniques. Tissue samples were placed in sterile microcentrifuge tubes and, to ensure macromolecule integrity, were snap-frozen in liquid nitrogen immediately after surgical resection and stored at −80 °C until use in our analysis. Sample handling was performed according to standard protocols to avoid contamination. The Ethics Committee of the Medical School of the University of Crete approved the current study and written informed consent was obtained from all the patients or their relatives.

5.1.2. Microscopic examination and histological classification of tumor samples

Hematoxylin- and eosin-stained slides for each tumor and normal specimen were prepared according to standard protocols. All tumor specimens were histopathologically examined by the same practitioner in order to determine tumor stage and grade. Histological grading of tumor samples was performed according to the 1973 WHO and the 2004 WHO/International Society of Urological Pathology (ISUP) classifications. Tumor stage was assessed according to the 2002 American Joint Committee on Cancer staging system. Normal adjacent tissue specimens were simultaneously examined in order to microscopically confirm the absence of contaminating tumor cells.

5.1.3. Post-operative patient treatment and follow-up

Patients with non-muscle-invasive disease were followed with periodic cystoscopic examinations, starting at 3 months after surgical resection of the tumors, as well as with intravesical treatment as indicated. Patients with muscle-invasive disease were treated with radical cystectomy with or without systemic chemotherapy. At a mean follow-up of 24 ± 3 months, 7 patients (23.3%) presented with disease relapse. In those with Ta/T1 vs T2-T3 tumors the frequency of relapse was 29.4% (5 of 17) vs 23% (3 of 13). In patients with non-muscle-invasive disease the progression rate was 11.1% and 22.2% for grades II and III disease, respectively. Disease relapse was assessed by biopsy in all cases.

5.1.4. Clinicopathological characteristics of the study cohort

Patients' mean age at the time of surgery was 72.2 ± 10.6 years (range 44–86). Ninety per cent (27/30) of the patients were males and 86.7% (26/30) of them were either current or former smokers at the time of diagnosis. All clinicopathological data of the patients included in the study were collected at diagnosis, microscopic examination and during follow-up and are listed in Table 5.1.1.

Table 5.1.1. Clinicopathological characteristics of the patients. (continued)

Characteristic	Bladder Cancer (%)
Cases (n)	30
Age (mean ± SD, years)	72.2 ± 10.6
Gender	
Male	27 (90.0)
Female	3 (10.0)
Tumor stage	
Ta-T1	18 (60.0)
T2-T4	12 (40.0)
Tumor grade	
II	10 (33.3)
III	20 (66.7)
Tumor type	
Papillary	23 (76.7)
Non-papillary	7 (23.3)
Recurrence	
Primary	24 (80.0)
Recurrent	6 (20.0)
Tumor sites	
One	22 (73.3)
Multiple	8 (26.7)
Therapy [a]	
Yes	18 (64.3)
No	10 (35.7)
Relapse [a]	
Yes	7 (36.8)
No	12 (63.2)
Survival	
Yes	22 (73.3)
No	8 (26.7)
Smoking habit	
Current smokers	18 (60.0)
Ex-smokers	8 (26.7)
Non-smokers	4 (13.3)

Table 5.1.1. Clinicopathological characteristics of the patients.

Characteristic	Bladder Cancer (%)
Occupational exposure [b]	
Yes	19 (63.3)
No	11 (36.7)
Family cancer history [a]	
Yes	9 (33.3)
No	18 (66.7)

[a] Data missing from some cases

[b] To chemicals, paints, pesticides, petroleum, ink, etc

5.1.5. Total RNA extraction from tissue samples

Total RNA was extracted from all tumor and normal samples according to the TRI Reagent® protocol (Ambion, Austin, TX). Tissue specimens were homogenized in TRI Reagent® using a power homogenizer. Following tissue homogenization, samples were centrifuged in order to remove insoluble material (e.g fat, polysaccharides) and high molecular weight DNA from the homogenates. The supernatants were transferred to fresh tubes and incubated at room temperature, followed by chloroform addition and centrifugation. Total RNA was precipitated from the supernatant with isopropanol, washed with 75% ethanol, resuspended in 50µl RNase-free water and stored at −80 °C until future use.

5.1.6. RNA spectrophotometry

For each sample, RNA concentration was determined by measuring its absorbance at 260 nm on a UV spectrophotometer. RNA spectrophotometry was also performed in order to assess RNA purity, as it is not uncommon for RNA samples to be contaminated

with high molecular weight DNA or other organic compounds. In order to determine RNA purity, the 260/280 nm absorbance ratio was measured for each sample. Ratios from 1.8 to 2.0 were accepted as suitable for our analysis.

5.1.7. Reverse transcription – cDNA synthesis

cDNA was synthesized by reverse transcription (RT) with the RETROscript® Kit (Ambion), using the two-step RT-PCR protocol. Two micrograms of total RNA, random decamers and nuclease-free water were incubated for 3 min at 85 °C for the heat denaturation of RNA secondary structures. Samples were immediately placed on ice for the addition of the remaining RT mix, which contained 10× RT Buffer, dNTP mix, 10 units RNase inhibitor and 100 units MMLV-Reverse Transcriptase. The final mix was incubated at 44 °C for 60min for the RT reaction to take place, followed by incubation at 92 °C for 10 min to inactivate the reverse transcriptase. cDNA samples were stored at −20 °C until future use.

5.1.8. Primer design

Primers were designed to detect the transcripts of *TP53*, *TP63*, *TP73* and of the isoforms TAp63, ΔNp63, TAp73 and ΔNp73 by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). All primers were designed using the PrimerSelect application of the Lasergene DNAStar 7.0 software (DNASTAR Inc., Madison, WI). All primer pairs were selected on the basis of the following general conditions:

- the two primers of each pair should be of equal or highly similar size, which should not exceed 24bp,
- the GC content of each primer should not be higher than 60%,
- the sequence of each primer should be such that the formation of self-dimers and/or cross dimers is avoided; in case this cannot be avoided, primer dimers

should be small in number and/or of low ΔG (Gibbs free energy) to prevent false positive product signals,

- primer melting temperature (T_m) should be highly similar for the primers of each pair,
- the size of the qRT-PCR product should not exceed 250bp and
- the primers of each pair should span an intron of at least 800bp to prevent false positive product signals, in case of potential contamination of cDNA sample with genomic DNA.

The aforementioned conditions are known to contribute to qRT-PCR reliability as far as specific product yield is concerned and were met to the highest extent possible, depending on the sequence and structure of each gene and the criteria of our study.

Primer pairs for the detection of *TP53*, *TP63*, *TP73* were designed to simultaneously detect both wild-type/full-length and mutant/truncated transcripts of the genes.

All mRNA-specific primers' sequences are listed in Table 5.1.2.

5.1.9. Quantitative Real-Time Polymerase Chain Reaction

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) is a laboratory technique based on the principles of classic PCR and applied for the amplification and quantification of a targeted DNA molecule. qRT-PCR is performed using either fluorescent dyes, which can bind to double stranded DNA, or fluorescence-labeled DNA probes. The accumulation of PCR product during thermal cycling, as detected by increased fluorescence, is monitored and reported using special software. Data collection is performed during the early exponential phase of the reaction and thus enables the accurate calculation of the initial template quantities. Moreover, the status of the experiment can be monitored as the run progresses, i.e. in real time. qRT-PCR has widely been used for the calculation of mRNA copy number in samples,

exhibiting increased sensitivity and reliability when compared to the conventional RT-PCR method.

The qRT-PCR experiments in our study were performed using SYBR Green chemistry. SYBR Green is a DNA-binding dye which nonspecifically intercalates into dsDNA, with absorption and emission maxima at 497 and 520 nm respectively. Although SYBR Green yields weak fluorescence signals when free in solution, its fluorescence increases up to 1,000-fold upon intercalation with dsDNA. As target DNA amplification proceeds in an exponential manner during qRT-PCR experiments, the number of SYBR Green molecules bound to dsDNA increases exponentially as well and so does the fluorescence signal measured by a detector. Also, this experiment type uses a standard curve to quantify the amount of target DNA present in samples. In addition, the thermal profile which is set up prior to the experiment includes a dissociation curve, thus enabling discrimination between specific and non-specific PCR products.

First, optimization assays were carried out to determine the optimal values for primer concentration and annealing temperature, in order to maximize the throughput and the specificity of the procedure. Each reaction was performed in a final volume of 20 μl containing 1 μl cDNA from tumor or normal samples, 2× Maxima™ SYBR Green quantitative real-time polymerase chain reaction Master Mix (Fermentas Life Sciences, Vilnius, Latvia) and 100 nM of each primer pair. After initial denaturation at 95 °C for 10 min, PCR was performed for 40 cycles comprising of denaturation at 95 °C for 30 s, annealing at 60 °C (for *TP53*, TAp63, ΔNp63, TAp73 and β-actin) or 62 °C (for *TP63, TP73* and ΔNp73) for 30 s and elongation at 72 °C for 30 s, followed by a melt-curve analysis, in which the temperature was increased from 60 to 95 °C at a linear rate of 0.2 °C/s. Data collection was performed both during annealing and extension, with two measurements at each step, and at all times during melt-curve analysis. A representative pool of all cDNA samples was diluted in a series of 6 two-fold dilutions and was run in the same plate in order to construct a standard curve for the quantification process. In each reaction, 2 non-template negative controls were included. All PCR experiments were performed on the Mx3000P real-time PCR thermocycler using the software version 4.1 (Stratagene, La Jolla, CA). For all samples, excluding those with no amplification plots or with dissociation curves indicating

primer-dimer accumulation or by-product formation, the expression of genes of interest was calculated as follows: the Ct values of all unknown samples were projected, with the help of the standard curve, to a relative mRNA quantity. This value was then divided by the same sample's β-actin mRNA value, for normalization purposes. The normalized values of each malignant bladder samples were then divided by the normalized values of their adjacent normal samples. A 2-fold increase (a value ≥ 2) or decrease (a value ≤ 0.5) in expression was considered biologically significant (overexpression or down-regulation, respectively).

5.1.10. Nucleic acid electrophoresis

Two per cent (w/v) agarose gels were prepared according to standard protocols for use in the electrophoresis of our qRT-PCR products (product sizes 70-246bp). Ethidium bromide was added at a concentration of 10ng/mL once the agarose mix had reached room temperature. Ethidium bromide is an intercalating agent which fluoresces when exposed to ultraviolet (UV) light, emitting a very strong signal after binding to DNA. Ten microliters of each qRT-PCR product were resolved in agarose gels and photographed on a UV light transilluminator, in order to verify the results of the melt-curve analysis.

5.1.11. Statistical analysis

p53, p63, p73, TAp63, ΔNp63, TAp73 and ΔNp73 mRNA levels were first evaluated by one-sample Kolmogorov-Smirnov goodness of fit test, in order to determine whether they follow a normal distribution pattern. Depending on the results, Pearson's correlation or the non-parametric Spearman rank correlation was used to determine their relation pair-wise and their association with continuous variables (e.g. age). Moreover, their association with categorical variables (e.g. tumor stage and grade) was determined by Student's t-test (after examining for equality of variances with Levene's test), or by its non-parametric equivalents Mann-Whitney U and Kruskal-Wallis H tests. Finally, the χ^2-test, using Fisher's exact test when indicated by the analysis, was used to

examine p53, p63, p73, TAp63, ΔNp63, TAp73 and ΔNp73 expression status in association with the various clinicopathological parameters after stratification. All statistical analyses were 2-sided and were performed with SPSS 11.5 (SPSS, Chicago, IL). Statistical significance was set at the 95% level (p-value < 0.05).

Table 5.1.2. Primer sequences, PCR annealing temperatures and amplicon sizes

Primer pair	Sequence (5' – 3')	Amplicon size (bp)	Annealing temperature (°C)
p53	GTGAGCGCTTCGAGATGTTC ATGGCGGGAGGTAGACTGAC	137	60
p63	CCTCCAACACCGACTACCCAG GCACCGCTTCACCACTCC	236	62
TAp63	AAGATGGTGCGACAAACAAGAT GGGACTGGTGGACGAGGA	155	60
ΔNp63	TGTACCTGGAAAACAATGCCCA GACGAGGAGCCGTTCTGAATCT	103	60
p73	GAAACGCTGCCCCAACCAC CACCACGACGCTCTGCCTG	142	62
TAp73	CTTCGACCTTCCCCAGTCA CAGATGTAGTCATGCCCTCCA	98	60
ΔNp73	ATCCTCGGCTCCTGCCTCACT CGCGGCTGCTCATCTGGTC	246	62
β-actin	CGGCATCGTCACCAACTG GGCACACGCAGCTCATTG	70	60

5.2. MATERIALS

5.2.1. Reagents

- TRI Reagent® (Ambion)
- Chloroform 100% (Sigma)
- Isopropanol 100% (Merck)
- Ethanol 100% and 75% (Merck)

5.2.2. Buffers

- DEPC-treated (RNase-free) dH_2O (Invitrogen)
- Nuclease-free water (Ambion)
- 10× RT Buffer (Ambion)
- Maxima™ SYBR Green qPCR Buffer (Fermentas)
- DNA electrophoresis buffer - 0.5× Tris-borate-EDTA (TBE): 0.09 TrisHCl, 0.09M boric acid, 2.5mM EDTA, pH 8.3
- DNA loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol and 40% glycerol

5.2.3. Enzymes

- RNase inhibitor (Ambion)
- MMLV-Reverse Transcriptase (Ambion)
- Maxima Hot Start *Taq* DNA Polymerase (Fermentas)

5.2.4. Nucleic acids and nucleotides

- Random decamers (Ambion)
- dNTP mix (Ambion)
- dNTPs (Fermentas)
- qPCR primers (Table 5.1.2)

5.2.5. Molecular Biology Kits

- RETROscript® Kit (Ambion)

5.2.6. Dyes

- SYBR Green I (Fermentas)
- ROX passive reference dye (Fermentas)
- Ethidium bromide (BDH)

5.2.7. Chemicals

- Agarose (Invitrogen)
- Boric acid (BDH)
- EDTA (BDH)
- Tris base (BDH)
- Bromophenol blue (Sigma)
- Xylene cyanol (Sigma)

- Glycerol (Invitrogen)

5.2.8. Standard solutions and buffers

Aqueous stock solutions and buffers were prepared in ddH_2O. The pH of buffers was determined with a pH meter, which was standardized before use according to the manufacturer's instructions. Buffers were stored at room temperature or according to the manufacturer's instructions.

5.2.9. Softwares

- NCBI BLASTn
- Lasergene DNAStar 7.0 (DNASTAR Inc)
- MxPro QPCR 4.1 (Stratagene)
- SPSS 11.5 (SPSS)

6. RESULTS

6.1. Expression analysis of p53, p63 and p73

In the present study, the mRNA expression profile of the p53 family genes (p53, p63 and p73) was examined using a qRT-PCR assay in normal and malignant samples from 30 patients diagnosed with bladder cancer. Beta-actin gene was used for normalization purposes (Figure 6.1.1).

Mean age at diagnosis was 72.2 years and the majority of patients (90%, n=27) were males. Histological examination revealed that at the time of diagnosis 60% of the malignant lesions were non-muscle-invasive (pathologic stages Ta-T1), whereas 40% were muscle-invasive (pathologic stages T2-T4). Additionally, 80% (n=18) of the patients presented with primary tumors and 20% (n=12) with recurrent disease. Statistical analysis revealed that the majority of non-muscle-invasive tumors were more likely to be moderately differentiated (Grade II) papillary lesions, whereas most muscle-invasive tumors were more likely to be poorly differentiated (Grade III), non-papillary lesions (p=0.002 and p=0.009, respectively). Additionally, disease recurrence was associated with multiplicity of tumors on the bladder wall (p=0.007). Finally, as expected, non-muscle-invasive carcinomas were associated with a favorable clinical outcome, whereas patients with muscle-invasive lesions, although treated with radical cystectomy, succumbed to the disease (p= 0.003).

p53 was overexpressed in 14/30 (47%) samples, downregulated in 3/30 (10%), while in 13/30 (43%) specimens its expression was normal. p63 and p73 expression pattern was slightly different. p63 and p73 were overexpressed in 12/30 (40%) and 13/30 (43%) specimens, respectively, whilst they were down-regulated in 8/30 (27%) and 7/30 (23%) samples, respectively. For both genes, 33 % of samples (10/30) had normal expression (Table 6.1.1, Figure 6.1.2).

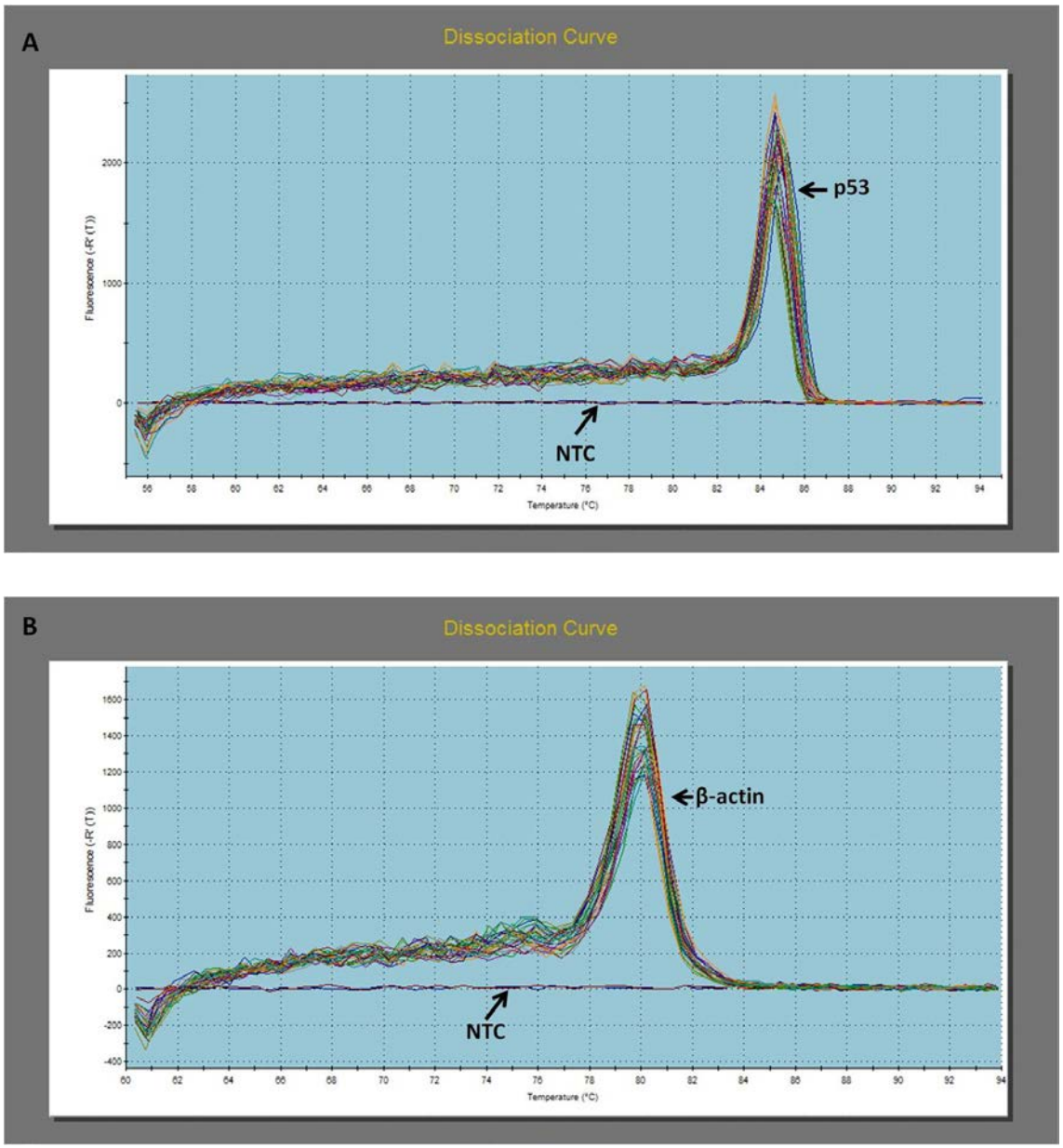


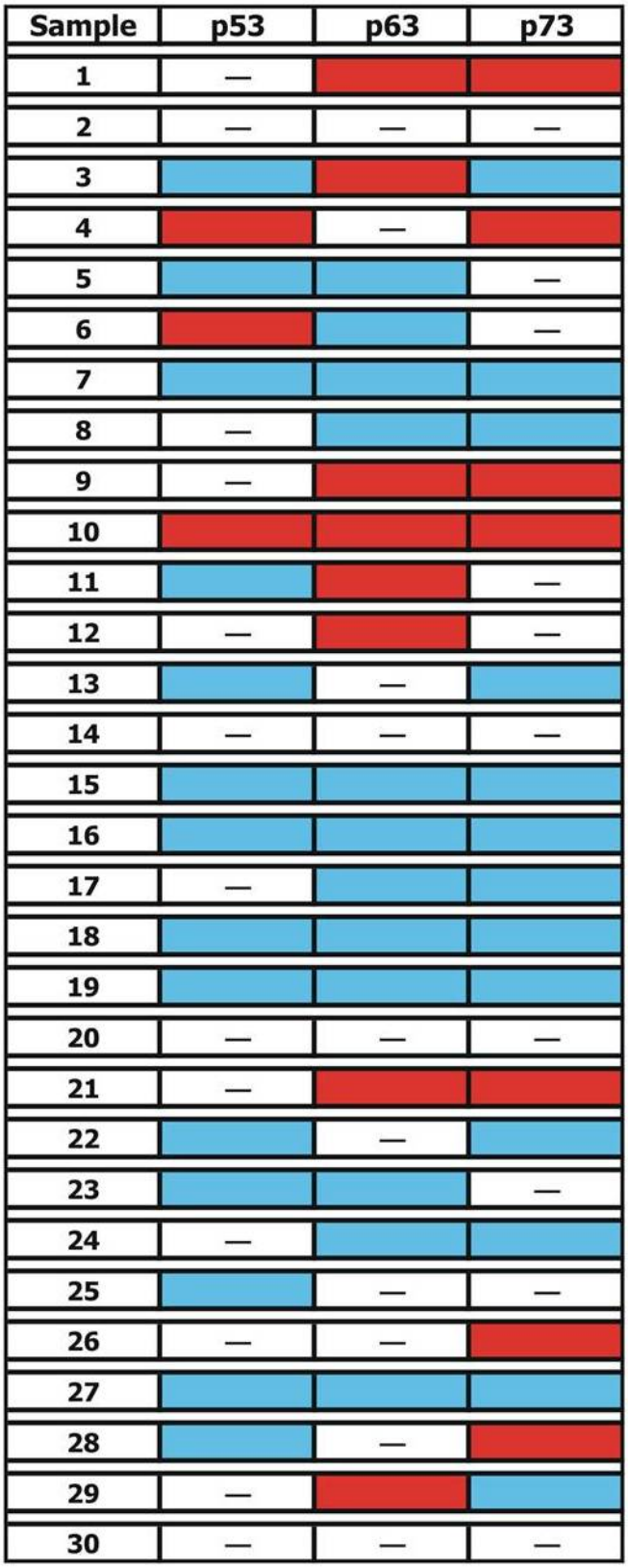
Figure 6.1.1: Quantitative Real-Time PCR. Dissociation curves of representative samples for **(A)** p53 and **(B)** β-actin. (NTC: negative control)

Further analysis showed that p53 exhibited a 1.9-fold overexpression in tumor specimens (from 0.99±0.13 in normal samples to 1.89±0.25 in tumor samples). Similarly, p63 was overexpressed by 3-fold in tumor specimens (from 1.04±0.26 in normal samples to 3.09±0.86 in tumor samples). Finally, p73 was overexpressed by 2-fold in tumor specimens (from 0.58±0.10 in normal samples to 1.19±0.21 in tumor samples; Figure 6.1.3).

With the Spearman rank correlation, we tested the co-expression patterns of the 3 examined genes in a pair-wise manner in the normal and malignant urothelium. This test examines whether 2 genes are up-regulated or down-regulated together (positive correlation), or whether when one gene is overexpressed and the other has a reduced expression (negative correlation). Results are displayed in Table 6.1.2. In the normal bladder epithelium, all members of the p53 gene family were co-expressed together ($p < 0.001$). However, in malignant urothelium, only the positive co-expression between p63 and p73 was maintained ($p=0.008$), whilst p53 was no longer co-expressed with either p63 or p73.

Table 6.1.1: Results of p53, p63 and p73 expression analysis in normal and malignant bladder tissue samples.

	Overexpression (%)	**Normal expression (%)**	**Reduced expression (%)**
p53	14/30 (47.0)	13/30 (43.0)	3/30 (10.0)
p63	12/30 (40.0)	10/30 (33.0)	8/30 (27.0)
p73	13/30 (43.0)	10/30 (33.0)	7/30 (23.0)



Sample	p53	p63	p73
1	—		
2	—	—	—
3			
4		—	
5			—
6			—
7			
8	—		
9	—		
10			
11			—
12	—		—
13		—	
14	—	—	—
15			
16			
17	—		
18			
19			
20	—	—	—
21	—		
22		—	
23			—
24	—		
25		—	—
26	—	—	
27			
28		—	
29	—		
30	—	—	—

Figure 6.1.2: Schematic representation of p53, p63 and p73 expression profile in our series of urothelial carcinoma tissue samples. (blue: overexpression; red; down-regulation; -: normal expression)

Statistical analysis revealed that p53 was up-regulated in non-muscle-invasive (pTa-T1) tumors compared to muscle-invasive (pT2-T4) ones (2.34±0.36 vs. 1.21±0.19, p=0.047). Moreover, significantly higher p63 mRNA levels were measured in malignant samples from patients > 75 years of age versus younger patients (4.56±1.61 vs. 1.63±0.59, p=0.022) and from patients with disease relapse compared to cancer-free patients (8.22±4.18 vs. 2.02±0.49, p=0.045). In addition, p63 expression significantly deviated from the normal pattern in papillary versus non-papillary lesions (3.61±1.16 vs. 1.55±0.53, p=0.026). Finally, p73 overexpression was observed in Grade III tumors compared with Grade II tumors, in patients presenting with one-site lesions (1.22±0.28 vs. 0.70±0.26, p=0.040) (Figure 6.1.4).

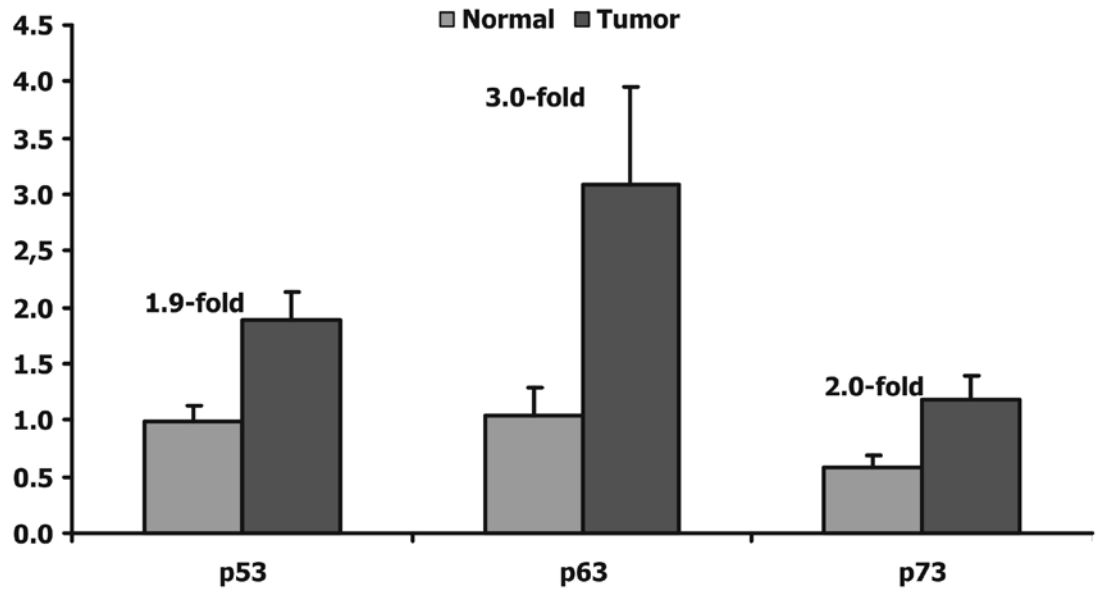


Figure 6.1.3: Bar chart depicting p53, p63 and p73 normalized expression in normal and malignant specimens, respectively. Floating numbers represent fold change between the two sample groups.

Table 6.1.2: p53, p63 and p73 pair-wise co-expression analysis in normal and malignant urothelium.

A. Normal Urothelium

		p53	**p63**	**p73**
p53	CC	1.000		
	P-value			
p63	CC	0.640	1.000	
	P-value	**<0.001**		
p73	CC	0.715	0.641	1.000
	P-value	**<0.001**	**<0.001**	

CC: Correlation Coefficient

B. Malignant Urothelium

		p53	**p63**	**p73**
p53	CC	1.000		
	P-value			
p63	CC	0.221	1.000	
	P-value	0.26		
p73	CC	0.290	0.493	1.000
	P-value	0.12	**0.008**	

CC: Correlation Coefficient

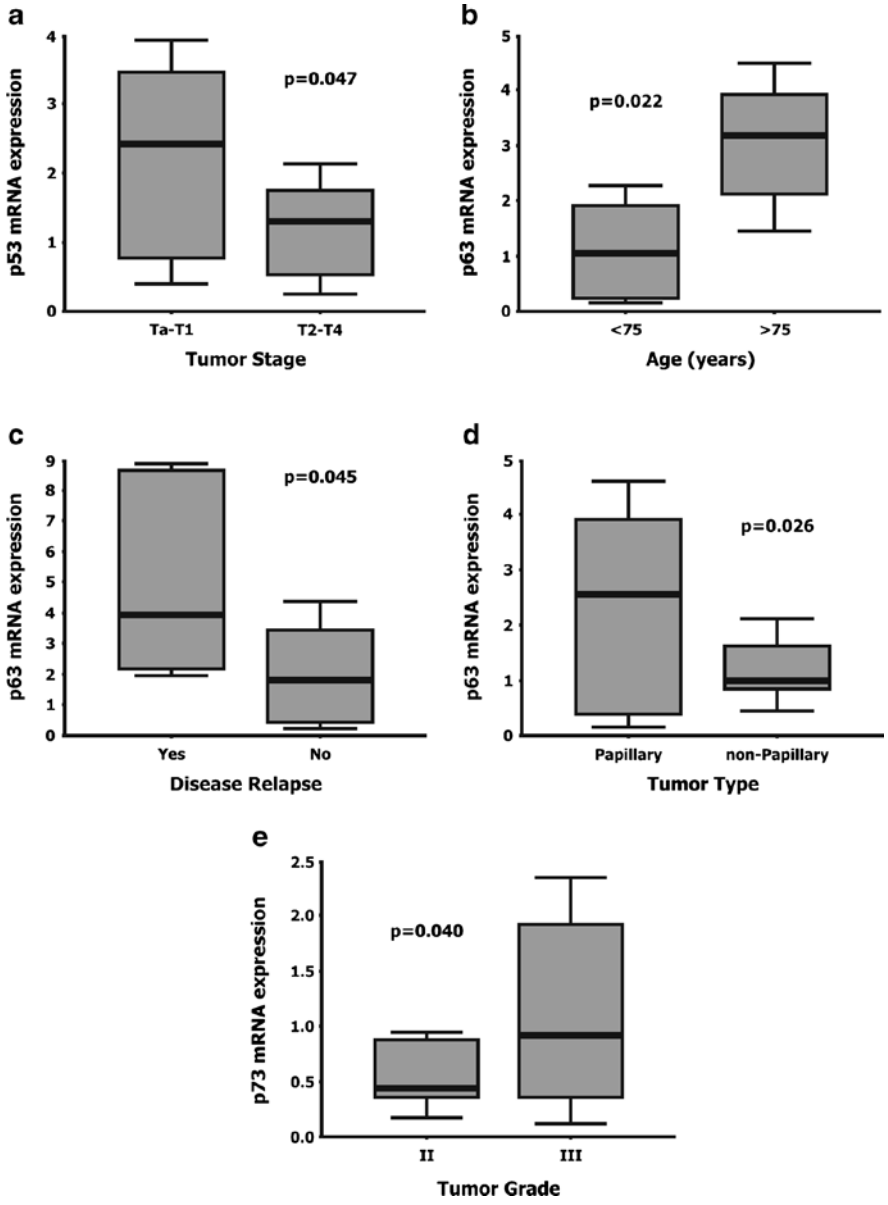


Figure 6.1.4: Box and whisker plots depicting statistically significant associations. **(a)** p53 was up-regulated in non-muscle-invasive (pTa-T1) compared to muscle-invasive (pT2-T4) tumors (p=0.047). **(b)** Significantly higher p63 mRNA levels were measured in malignant urothelial samples from patients >75 years of age (p=0.021) and **(c)** from patients presenting with disease relapse (p=0.045). **(d)** p63 expression deviated from the normal pattern in papillary versus non-papillary lesions (p=0.026). **(e)** p73 overexpression was observed in Grade III tumors compared to Grade II tumors, in patients presenting with one-site lesions (p=0.049). The thick line near the center of each rectangular box represents the median value, the bottom and top edges of the box indicate the 1st (Q_1) and 3rd (Q_3) quartiles, and the ends of the whiskers depict the 10th (P_{10}) and 90th (P_{90}) percentiles.

6.2. Expression analysis of TAp63, ΔNp63, TAp73 and ΔNp73

In the present study, the mRNA expression profile of p63 and p73 gene isoforms (TAp63 and ΔNp63; TAp73 and ΔNp73) was examined using a qRT-PCR assay in normal and malignant samples from 30 patients diagnosed with bladder cancer (Figure 6.2.1, Figure 6.2.2). Beta-actin gene was used for normalization purposes (Figure 6.1.1).

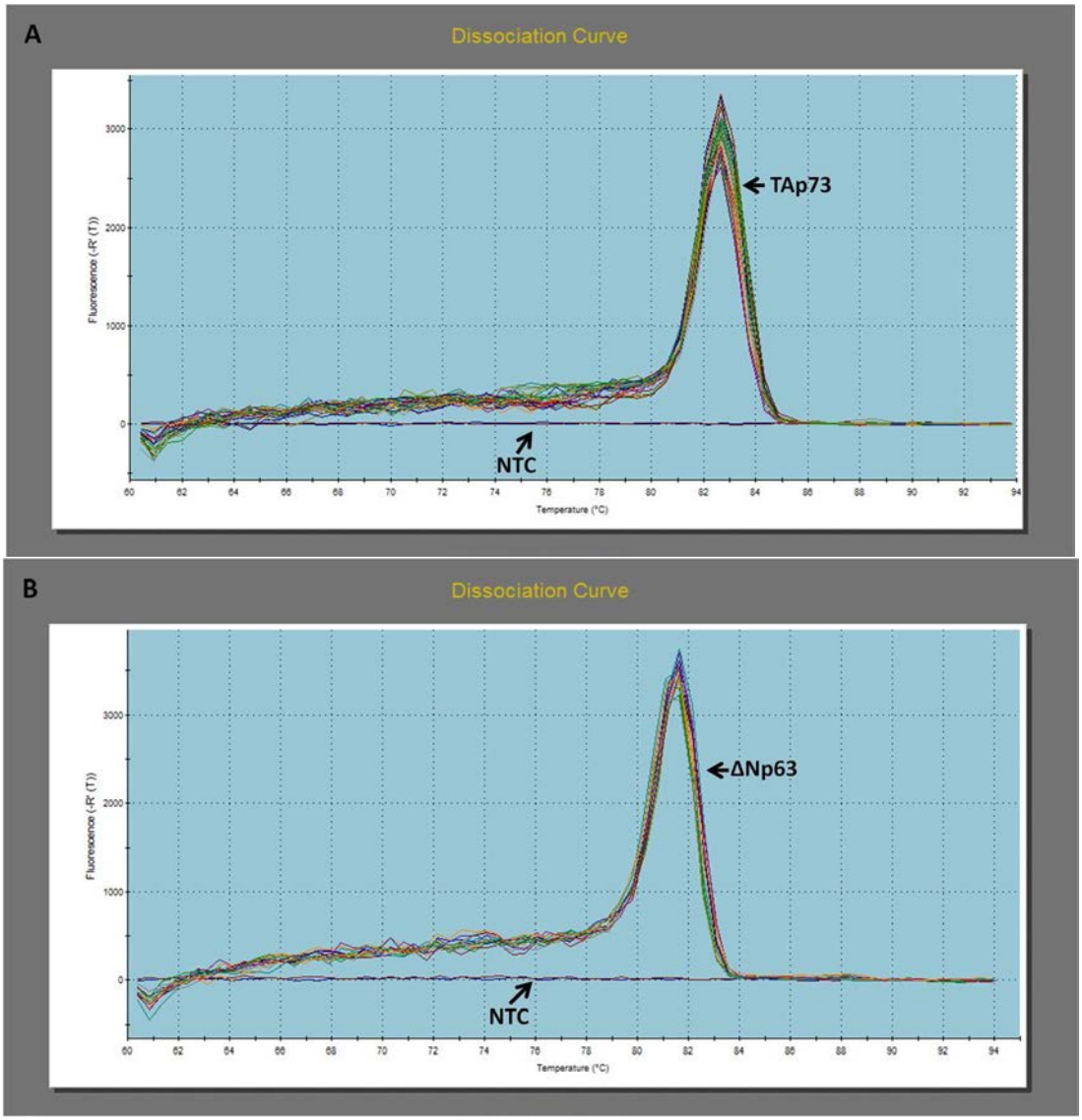


Figure 6.2.1: Quantitative Real-Time PCR. Dissociation curves of representative samples for **(A)** TAp73 and **(B)** ΔNp63. (NTC: negative control)

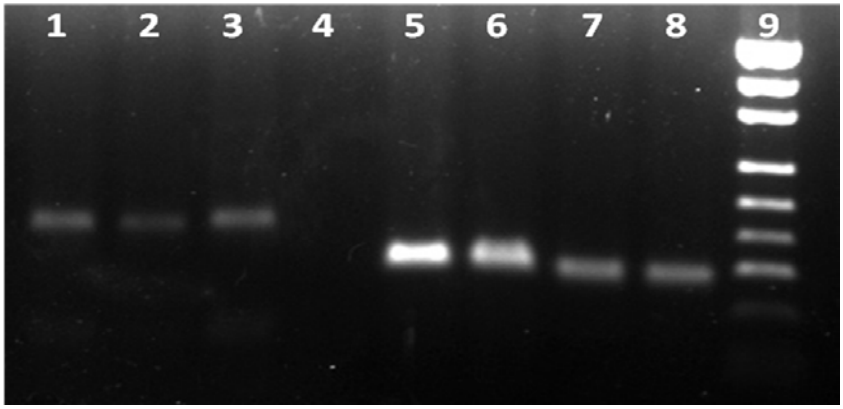


Figure 6.2.2: Agarose gel electrophoresis of representative qRT-PCR products. Lanes 1-3: TAp63 (155bp); lane 4: negative control; lanes 5 & 6: ΔNp63 (103bp); lanes 7 & 8: TAp73 (98bp); lane 9: DNA Ladder.

TAp63 isoform was overexpressed in 10/30 (33%) bladder cancer samples, downregulated in 4/30 (13%), while in 9/30 (30%) samples its expression was normal. Additionally, in 3/30 samples (10%) TAp63 was expressed only in tumor samples, while in 4/30 samples (13%) TAp63 was expressed only in normal urothelium. ΔNp63 was overexpressed in 15/30 (50%) and down-regulated in 4/30 (13%) bladder tumor specimens, while in 11/30 (37%) samples its expression was normal (Table 6.2.1 and Figure 6.2.3).

TAp73 isoform was overexpressed in 12/30 (40%) malignant samples, down-regulated in 9/30 (30%), while in 9/30 (30%) specimens its expression was normal. ΔNp73 was overexpressed in 11/30 (37%) and down-regulated in 2/30 (7%) samples. In 7/30 samples (23%) ΔNp73 was expressed only in tumor specimens, while in 4/30 samples (13%) ΔNp73 was only expressed in normal bladder tissue. Interestingly, in 3/30 (10%) samples, ΔNp73 was not expressed in either normal or its adjacent malignant specimen pair (Table 6.2.1 and Figure 6.2.3).

Table 6.2.1: Results of TAp63, ΔNp63, TAp73 and ΔNp73 expression analysis in normal and malignant bladder tissue samples.

	Overexpression (%)	Normal expression (%)	Reduced expression (%)	No expression (%)
TAp63				
Normal	—	27/30 (90.0)	—	3/30 (10.0)[a]
Tumor	13(10+3)/30 (43.3)[a]	9/30 (30.0)	4/30 (13.3)	4/30 (13.3)[b]
ΔNp63				
Normal	—	30/30 (100.0)	—	—
Tumor	15/30 (50.0)	11/30 (36.7)	4/30(13.3)	—
TAp73				
Normal	—	30/30 (100.0)	—	—
Tumor	12/30 (40.0)	9/30 (30.0)	9/30 (30.0)	—
ΔNp73				
Normal	—	20/30 (66.7)	—	10(7+3)/30 (33.3)[c,*]
Tumor	18(11+7)/30 (60.0)[c]	6/30 (20.0)	2/30(6.7)	7(4+3)/30 (23.3)[d,*]

[a] In 3 samples TAp63 was expressed only in tumor and not in the adjacent normal tissue.

[b] In 4 samples TAp63 was expressed only in normal and not in the adjacent tumor tissue.

[*] In 3 samples ΔNp73 was not expressed in either normal or tumor specimen.

[c] In 7 samples ΔNp73 was expressed only in tumor and not in the adjacent normal tissue.

[d] In 4 samples ΔNp73 was expressed only in normal and not in the adjacent tumor tissue.

Further analysis showed that TAp63 isoform exhibited a 2.9-fold overexpression in malignant samples (from 0.30±0.08 in normal samples to 0.87±0.25 in tumor specimens), while ΔNp63 was 3.2-fold overexpressed in cancer specimens (from 0.72±0.15 in normal samples to 2.26±0.52 in tumor specimens). Additionally, the ΔN/TA isoform ratio shifted 1.1-fold in favor of ΔNp63, from 2.37 in normal samples to 2.60 in tumor specimens. Interestingly, adding the expression levels of TAp63 and ΔNp63, the expression of total p63 increased on average 3.1-fold in tumor samples (from 1.02 in normal samples to 3.14 in tumor specimens) (Figure 6.2.4.A), a fold increase on par with our results concerning total p63 expression in bladder cancers.

Applying this analysis to p73 revealed that TAp73 isoform exhibited a 1.7-fold overexpression in tumor samples (from 3.42±0.73 in normal samples to 5.69±1.61 in tumor specimens), while ΔNp73 was 3.3-fold overexpressed in cancer specimens (from 0.77±0.22 in normal samples to 2.51±0.91 in tumor specimens). Additionally, the ΔN/TA isoform ratio shifted by 2.0-fold in favor of ΔNp73, from 0.22 in normal samples to 0.44 in tumor specimens. Interestingly, adding the expression levels of TAp73 and ΔNp73, the average expression of total p73 increased by 2.0-fold in malignant samples (from 4.19 in normal samples to 8.20 in tumor specimens) (Figure 6.2.4.B), a fold increase on par with our results concerning total p73 expression in bladder cancers.

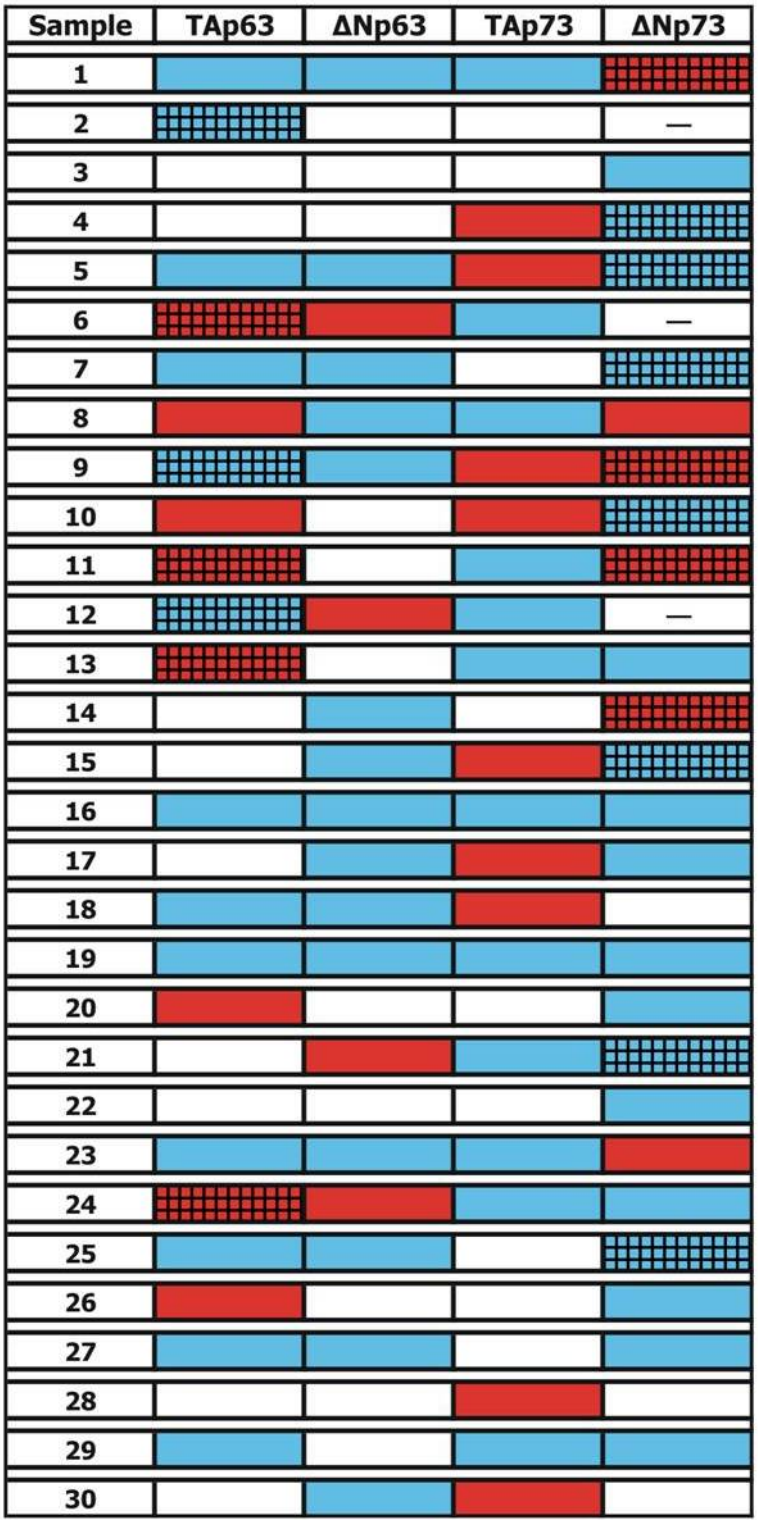


Figure 6.2.3: Schematic representation of TAp63, ΔNp63, TAp73 and ΔNp73 isoforms expression profile in our series of urinary bladder cancer tissue samples. (blue: overexpression; blue grid: expression only in tumor sample; red: down-regulation; red grid: expression only in normal sample; white: normal expression in both tumor and normal samples -: undetected expression in both tumor and normal samples)

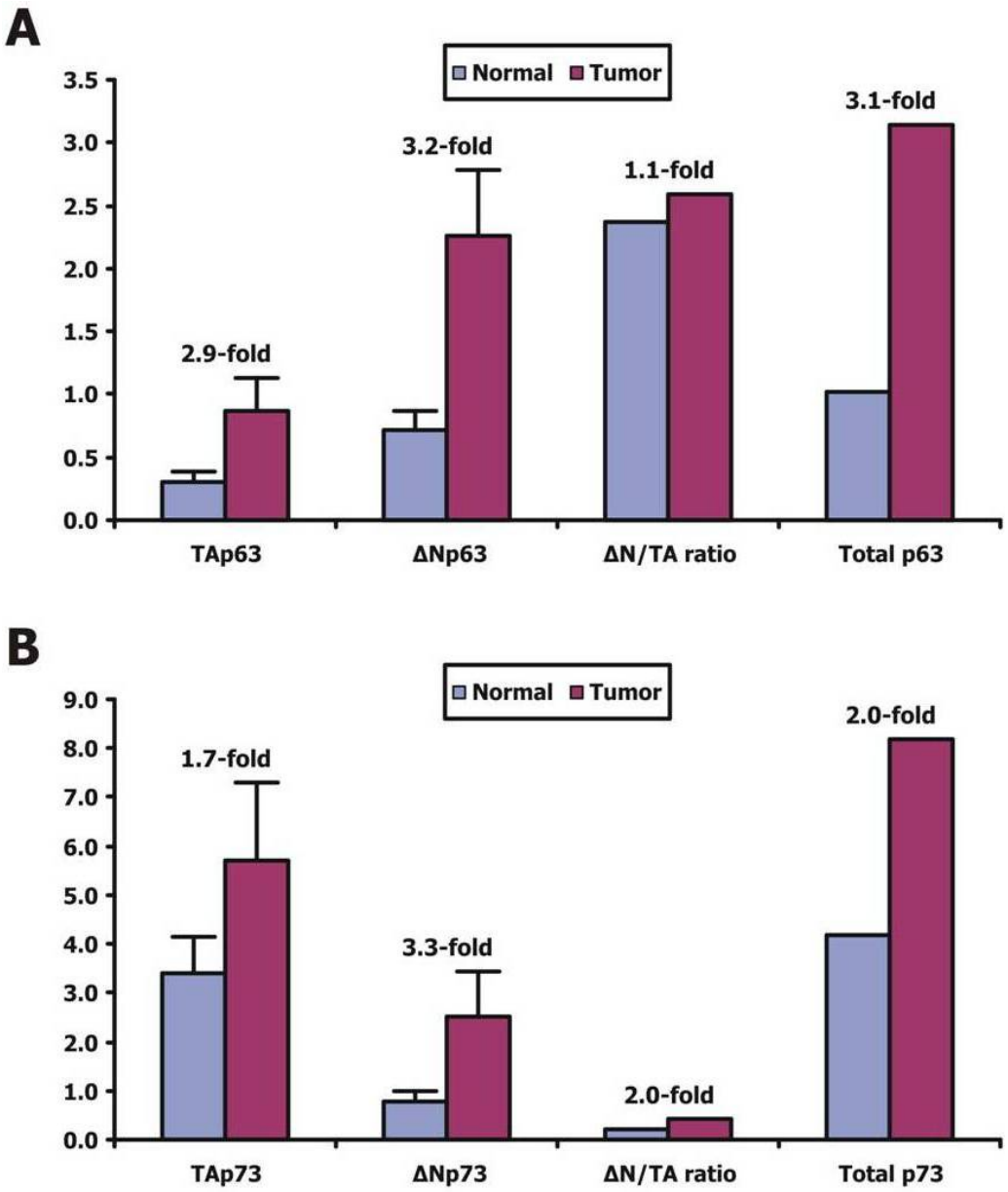


Figure 6.2.4: Bar chart depicting **(A)** TAp63 and ΔNp63 and **(B)** TAp73 and ΔNp73 normalized expression, as well as ΔN/TA expression ratio and total p63 **(A)** and p73 **(B)** expression, in normal and malignant bladder specimens, respectively. Floating numbers represent fold change between the two sample groups.

The results of the Spearman rank correlation are displayed in Table 6.2.2. In normal urothelium (Table 6.2.2.A) TAp73 was co-expressed with ΔNp73 (p=0.003). No other co-expressions were observed. However, in the malignant bladder epithelium (Table 6.2.2.B) this co-expression was lost. Interestingly, ΔNp63 was negatively correlated with TAp73 (p=0.006).

Statistical analysis revealed that ΔNp63 was up-regulated in tumor samples from bladder cancer patients older than 75 years old compared with younger (< 75 years old) patients (3.44±0.91 vs. 1.09±0.35, p=0.026), in non-muscle-invasive (pTa-T1) tumors versus muscle-invasive ones (pT2-T4) (3.12±0.80 vs. 0.97±0.29, p=0.019) and in patients receiving BCG as treatment instead of cystectomy (2.85±0.85 vs. 0.85±0.21, p=0.045) (Figure 6.2.5). Additionally, TAp73 mRNA levels were increased in patients with papillary tumors versus non-papillary ones (7.13±2.02 vs. 0.97±0.22, p=0.006) and in current smokers compared with non-smokers and ex-smokers combined (7.51±2.62 vs. 3.32±1.32, p=0.020) (Figure 6.2.6). No other statistically significant associations were observed.

Table 6.2.2. TAp63, ΔNp63, TAp73 and ΔNp73 pair-wise co-expression analysis in normal and malignant bladder epithelium.

A. Normal Urothelium

		TAp63	**ΔNp63**	**TAp73**	**ΔNp73**
TAp63	CC	1.000			
	P-value	–			
ΔNp63	CC	0.170	1.000		
	P-value	0.40	–		
TAp73	CC	0.187	-0.030	1.000	
	P-value	0.35	0.88	–	
ΔNp73	CC	0.445	0.012	0.630	1.000
	P-value	*0.056*	0.96	**0.003**	–

CC: Correlation coefficient.

B. Malignant Urothelium

		TAp63	**ΔNp63**	**TAp73**	**ΔNp73**
TAp63	CC	1.000			
	P-value	–			
ΔNp63	CC	0.337	1.000		
	P-value	*0.092*	–		
TAp73	CC	0.112	-0.491	1.000	
	P-value	0.59	**0.006**	–	
ΔNp73	CC	0.269	-0.050	0.110	1.000
	P-value	0.24	0.82	0.62	–

CC: Correlation coefficient.

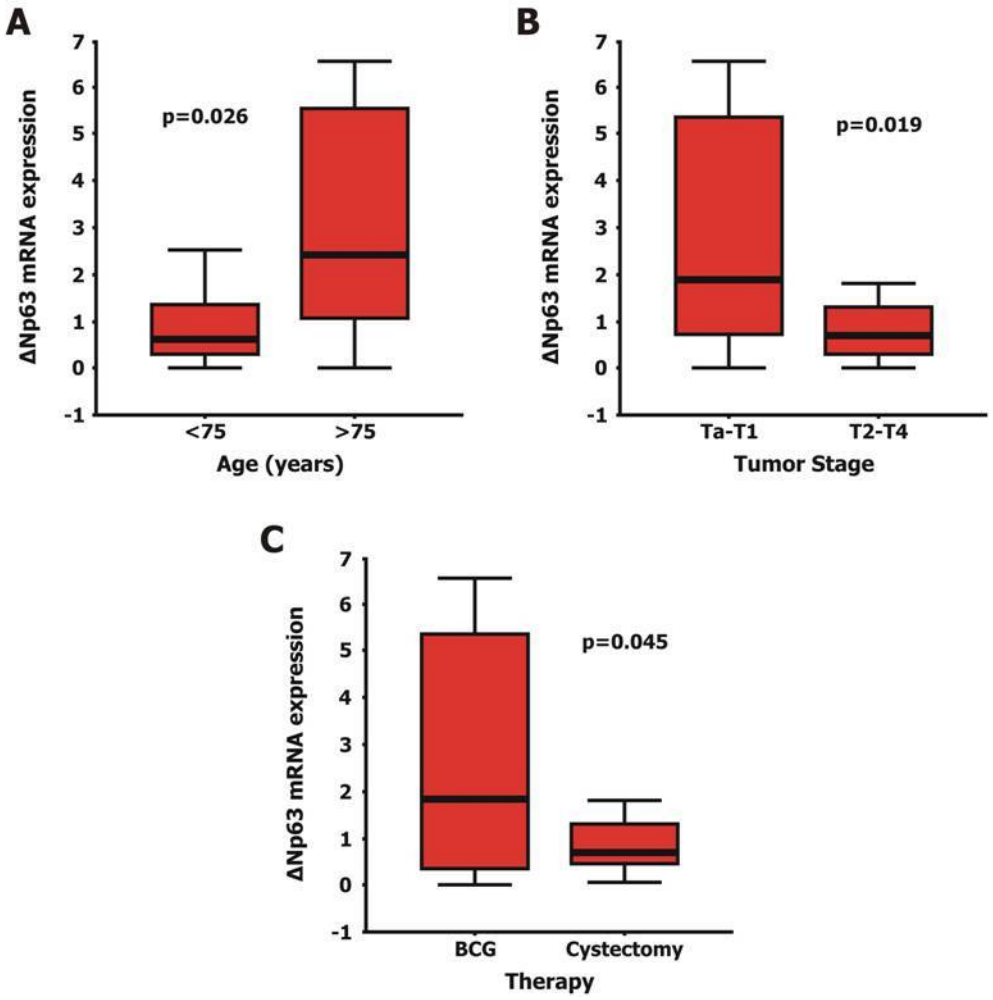


Figure 6.2.5: Box and whisker plots depicting statistically significant associations. **(A)** ΔNp63 expression was upregulated in tumor samples from older (> 75 years old) bladder cancer patients compared with younger (< 75 years old) ones (p=0.026), **(B)** in non-muscle-invasive (pTa-T1) tumors versus muscle-invasive ones (pT2-T4) (p=0.019) and **(C)** in patients that underwent BCG treatment instead of cystectomy (p=0.045). All values are presented as mean ± standard error of the mean (SEM). The thick line near the centre of each rectangular box represents the median value, the bottom and top edges of the box indicate the 1st (Q_1) and 3rd (Q_3) quartiles, and the ends of the whiskers depict the 10th (P_{10}) and 90th (P_{90}) percentiles. Statistical analysis was conducted with Student's t-test.

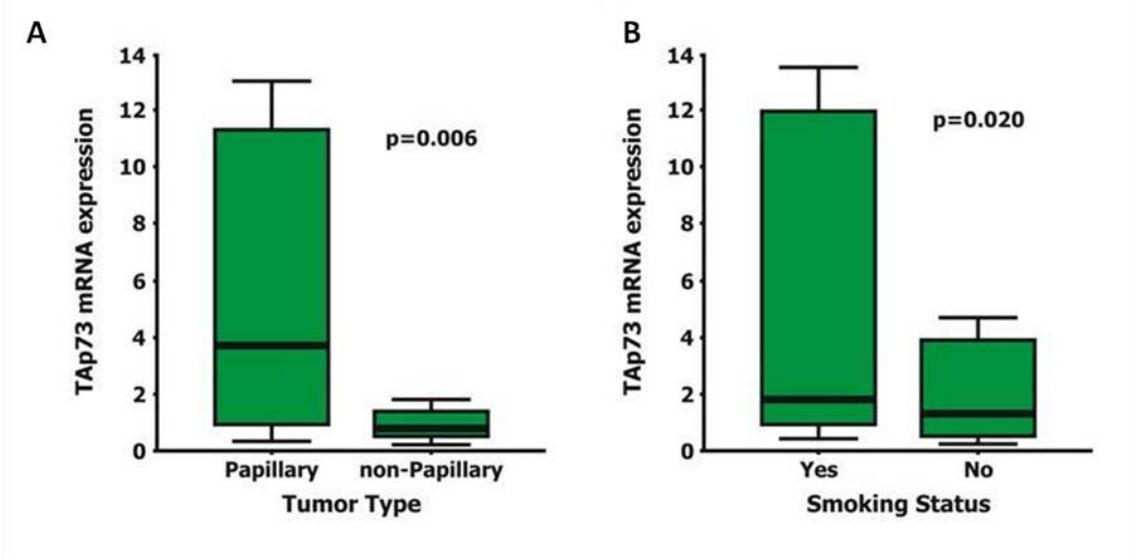


Figure 6.2.6: Box and whisker plots depicting statistically significant associations. TAp73 mRNA levels were increased **(A)** in patients with papillary tumors versus non-papillary ones (p=0.006) and **(B)** in current smokers compared with non-smokers and ex-smokers combined (p=0.020). All values are presented as mean ± standard error of the mean (SEM). The thick line near the centre of each rectangular box represents the median value, the bottom and top edges of the box indicate the 1^{st} (Q_1) and 3^{rd} (Q_3) quartiles, and the ends of the whiskers depict the 10^{th} (P_{10}) and 90^{th} (P_{90}) percentiles. Statistical analysis was conducted with Student's t-test.

7. DISCUSSION

In 2014, urinary bladder cancer ranks 11th among human malignancies worldwide [7]. It is estimated that 90-95% of bladder cancer cases are urothelial carcinomas, whereas the remaining 5-10% are mostly squamous cell carcinomas and adenocarcinomas [68].

Determination of tumor stage at diagnosis is crucial for prognosis and patient management, as the disease presents with two major phenotypic variants characterized by drastically different biological behavior [86]. Approximately 80% of newly diagnosed bladder cancer cases are non-muscle-invasive tumors of low grade, which have very good prognosis when treated early. The remaining 20% of the cases are muscle-invasive tumors with high metastatic potential, which ultimately confer an unfavorable patient outcome [8]. Both types of disease pose a significant economic burden and can seriously compromise patient quality of life. Non-muscle-invasive urothelial lesions are mostly not life-threatening, yet tend to recur within 5 years after initial diagnosis despite treatment and thus require lifetime surveillance. On the other hand, high-grade, muscle-invasive carcinomas are often lethal despite radical cystectomy and debilitating systemic therapy [8, 86].

Urothelial cancers harbor numerous genetic and epigenetic abnormalities, such as inactivation of tumor suppressor genes, activation of oncogenes and alterations in tumor microenvironment [86]. Mutations in the tyrosine kinase receptor *FGFR3* and protein overexpression are found in more than 70% of non-muscle-invasive tumors [96], whereas mutations that inactivate the tumor suppressor p53 protein characterize more than half of muscle-invasive lesions [88]. Intense research efforts are carried out to provide further insight into the molecular mechanisms driving malignant transformation of urothelial cells and aberrant biological behavior of the tumors. Identification of novel targets for more effective therapeutic interventions and, as well earlier diagnosis and more detailed prognosis is considered essential and remains the greatest challenge.

Since its discovery in 1979, the *TP53* gene has been the most extensively studied tumor suppressor and the p53 protein has been designated as "the guardian of the genome" [235]. *TP53* can be activated by a variety of stress signals, including DNA damage, hypoxia, oncogene activation, carcinogen exposure, mitotic spindle damage, ribonucleoside pool depletion, nitric oxide synthesis and starvation [236, 330]. The p53 protein subsequently accumulates and, depending on the severity of the stress imposed on the cell, either repairs the damage or drives the ablation of the cell harboring it. p53 is able to promote tumor suppression by activating transcription of genes involved in apoptosis, cell cycle arrest, senescence and autophagy (Figure 7.1) [330].

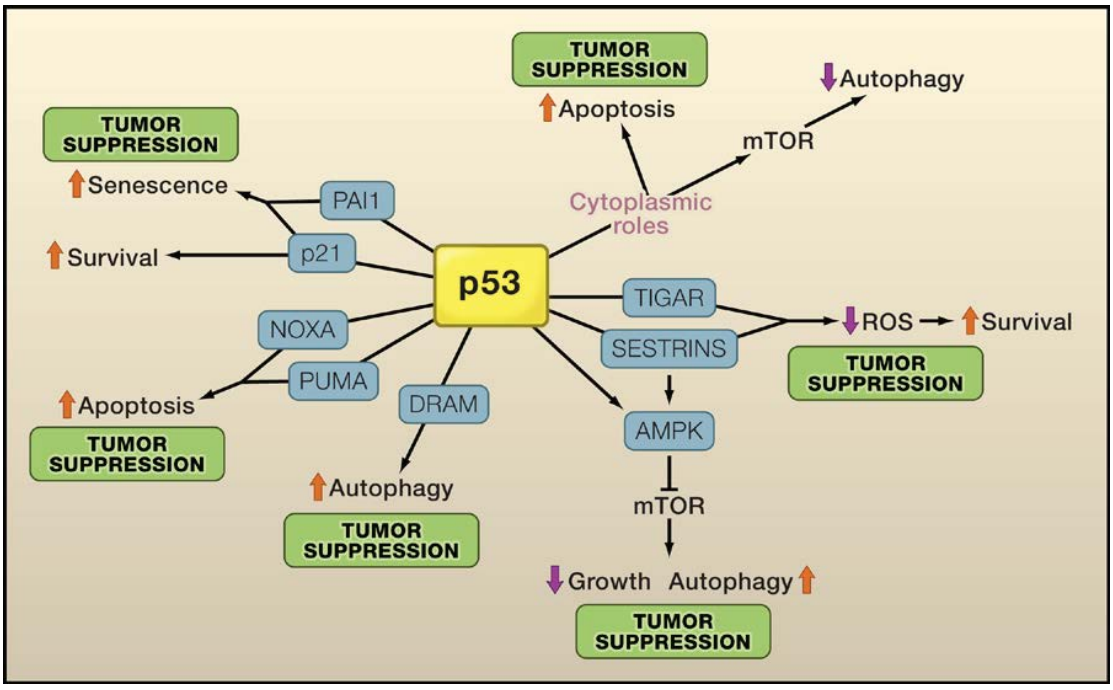


Figure 7.1: The control of cell survival, proliferation, and death by p53 is mediated by the regulation of expression of p53 target genes (blue boxes) in the nucleus and transcriptionally independent cytoplasmic functions of p53 [330].

Physiological function of p53 is abrogated by gene alterations in half of human malignancies [157]. Yet, the *TP53* homologues *TP63* and *TP73* are rarely mutated in tumors [159, 174-178, 185, 219]. p53-deficient mice develop multiple spontaneous

tumors [263], whereas p63- and p73-deficient mice are born with severe abnormalities in epithelial tissues and their respective appendages [264] and neurological and immunological defects [270], respectively.

Despite that *TP63* and *TP73* are not classic Knudson-type tumor suppressor genes, they both encode the full-length TAp63 and TAp73 isoforms, which co-operate with wild-type p53 in tumor suppression. Due to high homology with wild-type p53, especially in the DNA-binding domain, TAp63 and TAp73 can bind to p53-responsive elements and regulate transcription of genes with pro-apoptotic and anti-growth functions [251, 259]. On the other hand, usage of an alternative promoter generates the N-terminally truncated ΔNp63 and ΔNp73 isoforms, which exert tumorigenic functions by specifically interacting with p53 target sites [158, 252, 262] or by forming TA-ΔN hetero-dimers with reduced transactivation activity [184]. Most interestingly, ΔNp63 and ΔNp73 are indispensable for proliferation of undifferentiated epidermis [265] and for neuron maintenance [271], respectively (Figure 7.2).

More complexity has been added to the p53 family of transcription factors since it was identified that *TP53* can also produce the N-terminally truncated variants Δ40p53, Δ133p53 and Δ160p53 by alternative splicing, alternative initiation of translation and usage of an alternative promoter [165]. Like their p63 and p73 counterparts, some of these p53 isoforms can potentially inhibit p53 physiological function [240].

All p53 family genes give rise to multiple C-terminal variants via extensive alternative splicing. In total, *TP53*, *TP63* and *TP73* code for at least 12, 6 and 29 protein isoforms, respectively [164, 165].

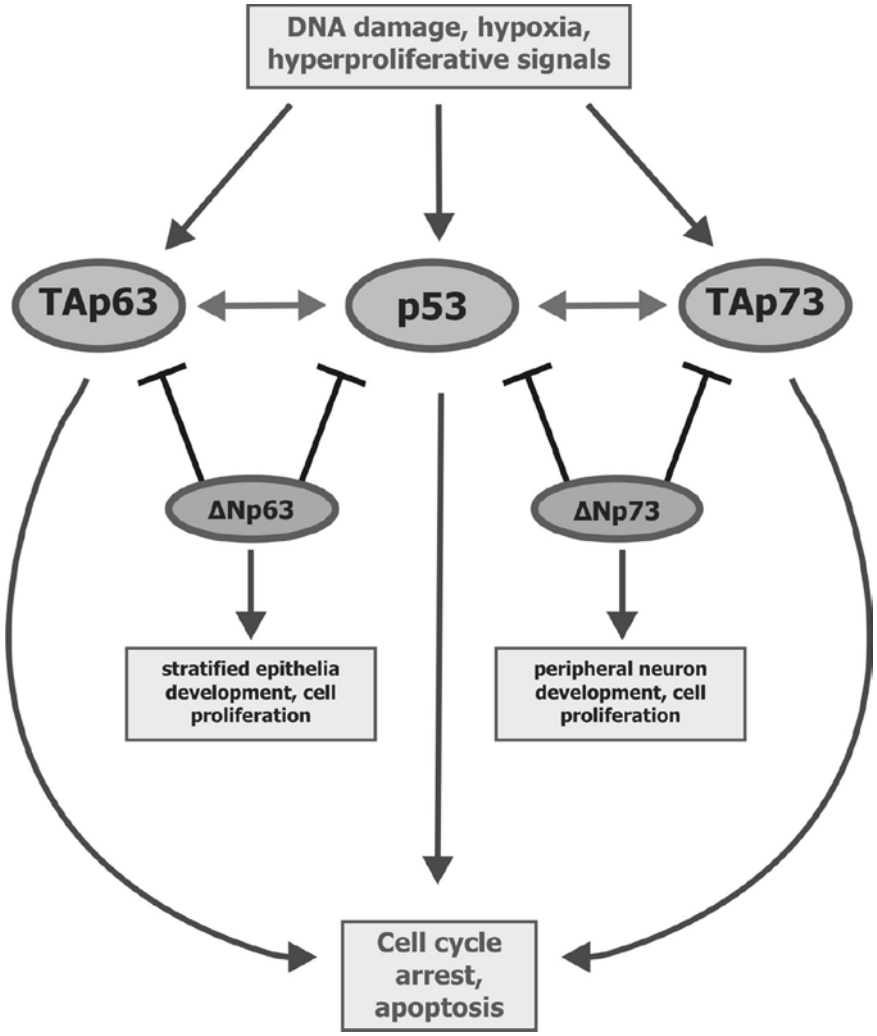


Figure 7.2: Roles of p53 family members in cell cycle control and development. Full-length TA isoforms cooperate with p53 in the induction of apoptosis, via transactivation of target genes. ΔN isoforms act as dominant negative inhibitors of p53 and their respective TA isoforms; they have oncogenic potential and also take part in developmental pathways.

In this study, the mRNA levels of p53, p63 and p73 were measured using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) in 30 urothelial carcinomas and in their normal adjacent tissues from patients who underwent surgery after presenting with either primary tumors or with recurrent ones. At the same time, the mRNA levels of TAp63, ΔNp63, TAp73 and ΔNp73 were measured. The results were analyzed and

correlations with clinicopathological parameters, as well as evidence for potential interactions between the three genes and/or their isoforms were investigated.

Normal p53, p63 and p73 mRNA levels were measured in non-cancerous bladder tissues, yet this expression pattern was disrupted in urothelial carcinomas.

p53 expression was increased by 1.9-fold in tumors when compared to normal tissues and p53 overexpression was detected in 47% of the samples. Statistical analysis revealed that p53 up-regulation was more frequent in non-muscle-invasive bladder tumors than in muscle-invasive ones ($p= 0.047$).

Dekairelle et al measured increased p53 mRNA levels in bladder transitional cell carcinomas (TCCs), in the majority of which p53 protein was also detected in high levels using immunohistochemistry. In this study, p53 positivity was noted in 47.8% of pTa-T1 tumors of grade 1 to 2 [331]. However, given that p53 mRNA is translated, our finding is in contrast with the results obtained in a large cohort study of 243 bladder cancer patients, in which p53 nuclear positivity was identified as a prognostic factor of disease progression, independently of stage and grade [293]. Similar results were obtained by Zigeuner et al, who reported an association of p53 overexpression with bladder tumors of advanced stage and grade, along with no p53 immunoreactivity in normal bladder and renal tissues [291]. In another study, altered p53 expression was identified in 56% of the bladder tumors analyzed and was associated with muscle-invasive disease [106]. Hitchings et al examined 78 pTa-T1 TCCs and found altered p53 expression in 45% of them [332]. In addition, it has been shown that p53 protein expression increases with disease stage [289, 290]. Goebell et al reported that p53 overexpression correlates with stage, grade and progression of bladder cancer in ≥ pT1 but not pTa tumors [102].

Also, *TP53* mutation status is crucial for prognosis of bladder tumors expressing p53. It has been reported that wild-type p53 patients have better overall survival and lower recurrence rates than the ones expressing mutant p53 [107, 109]. Mutant p53 protein has a prolonged half-life compared to the wild-type one and p53 positivity frequently correlates with the presence of mutations [333]. Although high concordances have been demonstrated between *TP53* gene alterations and p53 expression, various

studies reported detection of wild-type p53 in 19-47% of bladder tumors examined [100, 333, 334] and in one it was associated with poor prognosis [334]. Lu et al identified Mdm2 up-regulation in 7/9 urothelial carcinomas with p53 overexpression and absence of gene mutations, thus supporting the hypothesis that p53 protein accumulation could also be attributed to increased levels of its regulators [100]. Although Mdm2 does not transcriptionally activate *TP53*, it is known to stabilize p53 protein. Of note, George at el recently reported that mutations in exon 5 of the *TP53* gene correlate with wild-type protein status and may be associated with a more favorable patient outcome, at least in terms of disease recurrence, when compared to mutations in other exons [101]. In that context, we believe that data on the mutation status and protein expression of p53 and its regulators would be essential for a thorough evaluation of p53 expression profile and for a reliable interpretation of its association with clinicopathological parameters of our patient cohort.

Aberrant p53 protein expression has also been demonstrated in various types of human cancers and is mostly associated with the presence of mutations [204, 224, 278, 279, 282, 283]. Abnormal p53 expression has been associated with reduced overall and recurrence-free survival in hepatocellular carcinomas (HCCs) [279] and with tumor progression and lymph node metastasis in esophageal squamous cell carcinomas (SCCs) [281]. In prostate cancer, p53 positivity correlates with advanced stage, high Gleason grade, positive surgical margins and early biochemical tumor recurrence [282], whereas in non-small cell lung cancer (NCSLC) expression of mutant p53 may identify a more aggressive and life-threatening disease subset [283]. In ovarian carcinomas, expression of the p53β isoform was frequently detected in serous and poorly differentiated tumors and correlated with shorter recurrence-free survival in patients expressing wild-type p53. Expression of the newly identified, tumor-specific variant p53δ was also associated with poor response to chemotherapy [284]. Bourdon et al found that breast cancer patients expressing mutant p53γ had an overall survival as favorable as wild-type p53 patients, independently of estrogen receptor status. On the contrary, mutant p53 patients not expressing p53γ had a very poor prognosis [285].

Overall, it is becoming evident that data on isoforms expression, along with *TP53* mutation status, would be useful if not necessary for assessing the contribution of p53 in the progression of human cancers.

p63 expression was increased by 3.0-fold in tumors when compared to normal tissues and p63 overexpression was detected in 40% of the samples. Statistical analysis revealed that p63 up-regulation was more frequent in patients with disease relapse ($p= 0.045$) and in patients > 75 years of age ($p= 0.022$). Also, aberrant expression was significantly associated with papillary tumors ($p= 0.026$).

In our study, 7 patients (36.8%) presented with disease relapse during follow-up. Four of them were initially diagnosed with pTa-T1 tumors, two with pT2 and one with pT3 disease. For all patients diagnosed with non-muscle-invasive disease, at least one previous disease relapse had been recorded. In addition, 17 of 23 papillary carcinomas analyzed in our study were non-muscle-invasive (1 pTa and 16 pT1 lesions). Hence, the characteristics of our patient cohort confirm that most non-muscle-invasive bladder tumors are papillary lesions that frequently recur [86]. Before discussing the association of p63 expression with clinicopathological parameters in our patient cohort, we should note that p63 mRNA and protein levels were found to significantly correlate in transitional and squamous cell lines of the bladder [310], in urothelial tumors [312] and in hematological malignancies [301].

To our knowledge, no study so far has linked p63 overexpression with recurrent or papillary urothelial tumors exclusively. Yet, many studies have reported associations of p63 expression with bladder cancer stage and grade, thus indirectly supporting our results. Papillary urothelial neoplasms of low malignant potential express p63 protein in all cell layers, whereas p63 expression is gradually lost from non-muscle-invasive lesions to ≥ pT2 bladder tumors [290, 311]. Muscle-invasive urothelial carcinomas were found to express significantly lower p63 protein levels [289, 310], yet retention of p63 protein was correlated with adverse outcome and reduced overall survival in an analysis of 67 ≥ pT2 tumors by Choi et al [308]. Interestingly, Karni-Schmidt et al have previously reported a higher recurrence rate and lower recurrence free survival in patients with non-muscle invasive tumors not expressing p63 when compared to the

p63-positive cases [289]. These results point out a drastically different impact p63 expression may have on the progression of urothelial neoplasms depending on disease stage, most probably in co-operation with other proteins affecting tumor behavior. Reduced p63 protein expression has been associated with tumor grade and with progression from non-muscle-invasive to muscle-invasive disease in a set of pTa-T1 tumors examined by Urist et al [310]. Impaired p63 expression has also been associated with advanced tumor stage, lymph node metastasis and poor prognosis in TCCs of the bladder and the upper urinary tract [291, 312], whereas high p63 protein levels have been associated with good prognosis in bladder cancer patients [312].

Overall, p63 up-regulation appears to define a less aggressive subset of urothelial tumors with papillary morphology. Yet, more studies on large patient cohorts are required before identifying p63 total expression as a potential marker of early urothelial carcinogenesis.

Moreover, it should be noted that p63 mRNA and protein up-regulation have been linked to increased *TP63* gene copy numbers, especially in SCCs [177, 299]. Massion et al reported *TP63* genomic amplification in 88% of SCCs, 42% of large cell carcinomas and 11% of adenocarcinomas of the lung [299]. Similarly, Björkqvist et al had previously reported higher frequency of gains in chromosome 3q in lung SCCs than adenocarcinomas [335]. Increased *TP63* copy numbers were found in severe dysplasia and carcinomas in situ but not in lower grade lesions of the lungs. Importantly, *TP63* genomic amplification was associated with better survival of lung cancer patients [299]. To the best of our knowledge, evidence of *TP63* genomic amplification has not been reported in urothelial tumors.

p63 expression has been associated with clinicopathological parameters in other types of human cancers as well. In a study of 250 cervical carcinomas, p63 positivity was strongly associated with HPV 16 and loss of p63 expression coincided with transition from squamous to columnar or undifferentiated morphology of tumors [300]. p63 expression has been associated with better overall survival in patients with SCCs of the esophagus [336], whereas loss of p63 expression was correlated with progression of ductal breast carcinomas [304].

The observed association of p63 up-regulation with patient age will be discussed later in this chapter.

p73 expression was increased by 2.0-fold in tumors when compared to normal tissues and p73 overexpression was detected in 43% of the samples. Statistical analysis revealed that p73 up-regulation was more frequent in grade III tumors (p= 0.040).

These findings are mostly in agreement with the ones obtained in the few studies conducted so far on p73 expression in human urothelial tumors. Chi et al examined 45 primary bladder tumors and reported p73 mRNA overexpression in 40% of them, which was also strongly associated with tumor stage or grade [219]. Similarly, Schlott et al observed increasing p73 mRNA expression with tumor stage and grade in a study of 71 urinary TCCs [337]. Puig et al examined 154 TCCs, 8 TCC cell lines and one SCC cell line and reported a good correlation between p73 mRNA and protein expression. They also identified that most muscle-invasive tumors and invasive cell lines had lost p73 protein expression. p73 protein was detected in 57% of non-muscle-invasive and in 21% of muscle-invasive lesions and was significantly associated with disease stage [313].

In our subset of grade III tumors, 8 were non-muscle-invasive (1 pTa, 7 pT1), whereas 12 were muscle-invasive (8 pT2, 3 pT3, 1 pT4). Non-muscle-invasive, poorly differentiated tumors comprise a particular portion of urothelial tumors with variable prognosis. Approximately one third of the tumors will never recur or progress, another third will require deferred cystectomy and the last third will cause death of the patients [108]. Therefore, although our analysis did not produce significant results in this set of tumors, we believe that expression analysis of the *TP53* family genes in larger patient cohorts could provide results of potential prognostic value.

p73 abnormal expression has been correlated with aggressive tumor phenotypes in other malignancies as well. Yang et al noted higher incidence of p73 protein aberrant expression in stage IV neuroblastoma patients [186] and Nozaki et al detected higher p73 mRNA levels in meningiomas of advanced stage [193]. High p73 mRNA levels have also been measured in ependymomas [315]. Peters et al also detected significantly elevated p73 transcripts in acute myeloid leukemia patients and cell lines compared to

normal peripheral blood cells and noted a good correlation between p73 mRNA and protein in both human tissues and cell lines [232]. The latter finding was obtained in ovarian carcinomas by Ng et al, who also identified p73 mRNA overexpression in 4/7 ovarian carcinoma cell lines, 71% of invasive and 92% of borderline tumors [211], in agreement with other studies [227, 228]. Significantly elevated p73 mRNA levels have been detected in breast tumors [223]. Zaika et al examined 77 invasive breast carcinomas and 7 cell lines and found 5-25-fold p73 mRNA up-regulation in 38% of the tumors and 13-73-fold in 5/7 cell lines [197]. In another study, p73 mRNA overexpression was detected in 27% of breast cancers and was significantly associated with lymph node metastasis, vascular invasion and higher disease stage [222]. Sun examined 221 colorectal adenocarcinomas, including 58 cases of matched normal mucosa and primary and metastatic tumors. A gradual increase in the portion of cells expressing p73 protein was identified from 19% in normal tissues to 67% in primary tumors and 95% in metastatic tumors and p73 overexpression was significantly associated with poor disease outcome [280]. Similarly, p73 overexpression correlated with perineural/vascular invasion in HNSCCs [294].

Our analysis also revealed that p53, p63 and p73 are co-expressed in normal urothelium and that this expression pattern is disrupted in the malignant bladder, where only p63 and p73 are co-expressed. p53 expression and/or mutation has been found not to correlate with p63 and/or p73 expression not only in bladder tumors [219, 234], but also in breast carcinomas [197] and lung tumors [327]. On the other hand, p63 and p73 protein co-expression was identified in HNSCCs [294]. Our results indicate that p63 and p73 exert their contribution to the malignant transformation of bladder epithelium independently of p53. Earlier studies in human keratinocytes and prostate and breast cancer cells have also shown that p63 and p73 respond to different elements, including p53, in terms of their transcriptional regulation [243]. In that context, we believe that respective studies in urothelial cells, both normal and cancerous, would be of great interest and could shed light on the mechanisms governing *TP53* family genes regulation in the human bladder.

The TAp63, ΔNp63, TAp73 and ΔNp73 isoforms were mostly expressed at normal levels in the normal bladder samples we analyzed, with some exceptions that will be

discussed later in this chapter. On the contrary, all isoforms were aberrantly expressed (up- or down-regulated) in the majority of urothelial carcinomas. TAp63 and ΔNp63 were aberrantly expressed in 17/30 and 19/30 bladder tumors, respectively. TAp73 and ΔNp73 were aberrantly expressed in 21/30 and 20/30 bladder tumors, respectively.

TAp63 expression was increased by 2.9-fold in tumors when compared to normal tissues. TAp63 overexpression was detected in 33% of the samples, whereas TAp63 was down-regulated in 13% of the samples. These findings are not in complete accordance with the ones reported in previous studies. Reduced TAp63 mRNA expression has been detected in 53.2% and 100% of urothelial tumors [307, 309], whereas in other studies TAp63 were undetectable in bladder tumors and cell lines [310, 311], except in one non-muscle-invasive cell line [310]. Park et al detected reduced TAp63 mRNA levels in 28% of pTa-T1 tumors and in 81.8% of ≥ pT2 tumors and identified a significant correlation of TAp63 down-regulation with tumor stage and grade. Also, TAp63 expression correlated with patients cumulative survival after operation [307]. Compérat et al detected significantly fewer TAp63 transcripts in pTa-T1 tumors when compared to normal bladder tissues [309].

In our samples, TAp63 expression was tumor-specific in 3 tissue pairs, whereas in 4 tissue pairs TAp63 was detected only in normal but not in tumor samples. TAp63 and ΔNp63 tumor-specific expression has previously been reported and methylation was suspected as a potential mechanism underlying deregulated p63 isoforms expression in 3/4 bladder cancer cell lines examined [308]. Yet, these findings were not confirmed by later studies in bladder or other carcinomas. Vakonaki et al identified no CpG islands in the P1 and P2 promoters of the *TP63* gene [338]. Hence, promoter methylation is not the cause of TAp63 down-regulation in our samples. In addition, chromosome gain rather than loss has been reported to occur in 3q, where the *TP63* gene is located [177, 299]. To our knowledge, loss of heterozygosity has also not been reported for 3q or *TP63* in particular in bladder tissues. Moreover, *TP63* mutations in human cancers are extremely rare [174-178]. *TP63* mutations associated with developmental disorders result in truncated proteins rather than in absence of protein expression [179-181]. Therefore, we hypothesize that TAp63 transcription could be

regulated by acetylation. Evidence for ΔNp63α acetylation has been reported in SCC cell lines and normal human keratinocytes [339]. LeBoeuf et al also reported maintained total p63 expression in ectoderm deficient of histone deacetylase genes (*Hdac1/2*) [340]. Although micro RNAs (miRNAs) have been described as post-transcriptional ΔNp63 regulators [251], Papagiannakopoulos et al noted significant TAp63 protein down-regulation in cervical cancer cells after ectopically expressing miR-21 [341]. Thus, TAp63 down-regulation could also be attributed to transcription repression by miRNAs.

ΔNp63 expression was increased by 3.2-fold in tumors when compared to normal tissues and ΔNp63 overexpression was detected in 50% of the samples. Significantly higher ΔNp63 mRNA levels were measured in non-muscle-invasive tumors (p=0.019), in patients receiving *Bacillus Calmette-Guérin* (BCG) treatment (p=0.045) and in patients > 75 years of age (p= 0.026).

Therapeutic approach with BCG is frequently applied in low- and high-grade non-muscle-invasive tumors of the bladder [76, 77]. Of the 9 patients who received BCG treatment in our cohort, 8 were diagnosed with pT1 bladder tumors of grade II or III and 1 was diagnosed with pTa tumor of grade III. Therefore, the first two associations we identified basically constitute the same finding.

ΔNp63 is expressed at various levels in normal urothelial cells, bladder cancer cell lines and solid tumors [310]. In accordance with our results, ΔNp63 mRNA was up-regulated in 63.8% and 100% of the tumors analyzed in two studies [307, 309], although no statistically significant association with stage and/or grade was identified. On the other hand, Karni-Schmidt et al detected ΔNp63 protein in 19.7% of non-muscle-invasive tumors and in 41.8% of muscle-invasive ones. Especially within the subset of ≥ pT2 urothelial carcinomas, it was found that ΔNp63-positive patients had shorter survival than ΔNp63-negative patients [289]. Similarly, Koga et al reported that impaired ΔNp63 expression characterizes biological aggressiveness of urothelial neoplasms and is associated with reduced β-catenin expression, which is possibly linked to poor prognosis [311, 312].

In our study, the ΔN/TA isoform ratio shifted 1.1-fold in favor of ΔNp63, from 2.37 in normal samples to 2.60 in tumor specimens. Similar results were obtained by Vakonaki et al, who also detected TAp63 and ΔNp63 up-regulation in 20 endometrial tumors compared to normal tissues. They also identified significant associations of TAp63 with obesity and late menopause. Interestingly, decreased TAp63 and increased ΔNp63 mRNA levels correlated with grade I/II endometrial tumors [338]. Increased TAp63 and ΔNp63 transcripts have also been measured in HNSCCs [298], gastric cancer precursor lesions [208], normal breast and benign breast lesions [305, 342]. de Biase et al found TAp63 overexpression in 75% of basal-like breast carcinomas [342]. Interestingly, TAp63 overexpression has been detected in high-grade follicular lymphomas [301], but the significance of this finding is as of now elusive. Marchini et al reported comparable TAp63 and ΔNp63 mRNA levels in 83 stage I ovarian tumors, yet noted an increase of ΔNp63 over TAp63 by 77.41-fold and by 11.48-fold in 86 stage III cancers and 7 ovarian cancer cell lines, respectively. The ΔN/TA ratio was significantly associated with disease stage, grade, histology and residual tumor [343].

Thurfjell et al, who found ΔNp63 up-regulation in HNSCCs compared to normal tissues, suggested that high levels of ΔNp63 may represent maintained expression by the basal cells rather than an overexpression during tumorigenesis [298]. Such an explanation cannot be ruled out for our finding of ΔNp63 up-regulation in urothelial tumors, since ΔNp63 is the predominantly expressed isoform in the progenitors of basal, intermediate and umbrella cells of adult urothelium [268] and is known to play a key role in bladder morphogenesis [269].

Data from various studies bestow an oncogenic role upon ΔNp63, as they show that it is required for cell survival. Ablation of p63 from SCCs results in apoptosis, as it is indicated by increased PUMA and NOXA expression [297]. ΔNp63 has indeed been shown to exert anti-apoptotic effects by either competing with TAp73 for binding to promoters of pro-apoptotic genes or directly binding to TAp73 to inhibit its function [297, 344]. Interesting results were obtained in a study of 37 primary invasive breast tumors. ΔNp63 was up-regulated, while TAp63 was detected at lower levels. TAp73 was also up-regulated and co-expressed with ΔNp63 *in vivo*. It was shown that ΔNp63 can promote survival of breast cancer cells by binding to TAp73. Six hours after

cisplatin treatment, c-Abl-dependent TAp73 phosphorylation on Tyr99 was induced and ΔNp63/TAp73 complexes were found to dissociate, resulting in TAp73-mediated activation of pro-apoptotic molecules [345]. The oncogenic potential of ΔNp63 was further supported in a study on HNSCC and osteosarcoma cell lines. ΔNp63α mRNA expression correlated with Heat-Shock Protein 70 (HSP70) mRNA expression and the two proteins co-expressed and were colocalized in epithelial tumors. Furthermore, ΔNp63α was found to up-regulate expression of HSP70 mRNA by interacting with transcription factors binding to its promoter. HSP70 chaperones may exert tumorigenic functions by sequestering wild-type p53 in the cytoplasm of cancer cells [346]. ΔNp63 can also repress transcription of pro-apoptotic genes like *PUMA* by forming complexes with histone deacetylases HDAC1 and HDAC2 in SCC cells. Cisplatin treatment induced dissociation of the p63/HDAC complex from the *PUMA* promoter, leading to histone acetylation, *PUMA* activation and apoptosis [347].

Interestingly, ΔNp63 appears to inhibit metastasis in bladder cancer cells. In low-grade urothelial cells of epithelial morphology expressing only ΔNp63α protein, knockdown of endogenous p63 resulted in N-cadherin up-regulation, Shc adaptor protein recruitment, ERK signaling activation and ultimately to potentiated cell motility, excretion of MMP-9 and invasion. On the other hand, exogenous expression of ΔNp63α in high-grade urothelial cells of mesenchymal morphology resulted in down-regulation of N-cadherin expression and ERK signaling, thus suppressing invasion. Fukushima et al also demonstrated loss of ΔNp63α protein expression and N-cadherin up-regulation in patients with progression from low-grade, non-muscle-invasive to muscle-invasive disease [348]. Tran et al also reported ΔNp63α, E-cadherin and miR-205 co-expression in bladder cancer cell lines and primary carcinomas. In bladder cancer cells, ΔNp63α was found to regulate epithelial-mesenchymal transition (EMT), which is characterized by loss of E-cadherin. ΔNp63α knockdown in bladder cancer cells of epithelial morphology induced expression of ZEB1/2, which are transcriptional repressors of E-cadherin, and decreased the expression of miR-205, which prevents EMT by repressing ZEB1/2. ΔNp63α overexpression in bladder cancer cells of mesenchymal morphology induced miR-205 and suppressed ZEB1/2, thus inhibiting EMT [349].

Overall, it appears that ΔNp63 contributes to squamous cell carcinogenesis by inhibiting apoptosis and promoting cell survival, yet may suppress invasion in urothelial carcinomas. Our results on ΔNp63 up-regulation in non-muscle-invasive tumors are more consistent with the latter property of ΔNp63, yet we believe that investigation of pro-apoptotic gene regulation by ΔNp63 in malignant urothelial cells would be of great interest.

In our patient cohort, ΔNp63 up-regulation, along with total p63 up-regulation, were found to be associated with late disease onset (> 75 years of age) ($p=0.026$ and $p=0.045$, respectively). To our knowledge, association of p63 expression with patient age has not been reported so far. After intense search of the literature, we found a meta-analysis of age-related gene expression profiles using datasets from mice, rats and humans. According to the results, genes involved in inflammation and immune response and genes associated with the lysosome are overexpressed with age [350]. In study by Yang et al, ΔNp63α was identified as a major transcription factor which co-operates with NF-κB to drive a broad gene program in SCC cell lines. Its overexpression in cell lines and transgenic mice resulted in increased epithelial proliferation, enhanced cytokine expression and diffuse inflammation [351].

In contrast to the role of ΔNp63 in promoting cell survival, TAp63 promotes cell cycle arrest and apoptosis. TAp63α is expressed at high levels in oocytes and is required for the protection of the female germline from genotoxic stress during meiotic arrest. Suh et al reported that oocytes die within 5 days after exposure to ionizing radiation. Oocytes from TAp63-null mice were resistant to apoptosis, while p53-null oocytes were sensitive to apoptosis, indicating that TAp63 and not p53 is indispensable for these effects in oocytes [352]. It was later shown that cisplatin treatment induces c-Abl-dependent TAp63 phosphorylation, which activates TAp63 to promote apoptosis [353]. Exogenous expression of TAp63α in p53-null human lung adenocarcinoma cells can induce apoptosis by transcriptionally activating *PUMA* and *NOXA* [353]. In the same cells, inhibition of TAp63 by mutant p53 was found to promote cell invasion into Matrigel. Knockdown of TAp63 by siRNA in p53-null cells resulted in random migration, a finding which further supports a potential role of TAp63 in the inhibition of metastasis [354]. These lines of evidence indicate that TAp63 may be expressed in

tumors, yet may not be able to exert its pro-apoptotic function. Although authors often note that these results are context-dependent, we think that respective studies in normal and malignant urothelia would produce useful information on the tumor suppressive role of TAp63.

In our study, TAp73 expression was increased by 1.7-fold in tumors when compared to normal tissues and TAp73 overexpression was detected in 40% of the samples. ΔNp73 expression was increased by 3.3-fold in tumors when compared to normal tissues and ΔNp73 overexpression was detected in 37% of the samples.

To our knowledge, there has been only one other study so far reporting TAp73 and ΔNp73 expression not in solid bladder tumors but in cell lines. Puig et al detected elevated TAp73α transcripts in 3/8 TCC cell lines and reported a good correlation between mRNA and protein levels. One TCC cell line expressed only ΔNp73, as did one SCC cell line also examined, and TAp73 was not detected in 4 TCC cell lines. ΔNp73 was expressed in all cell lines [313].

TAp73 up-regulation was more frequent in papillary tumors (p=0.006). As has already been mentioned, the majority of papillary carcinomas analyzed in our study (17/23) were non-muscle-invasive lesions. Yet, we can identify only an indirect link of TAp73 expression with urothelial tumors characterized by fairly good prognosis. Studies on larger patient cohorts are required to establish TAp73 as a potential biomarker of papillary morphology or favorable patient outcome.

Also, TAp73 up-regulation was more frequent in patients who were smokers at the time of diagnosis (p=0.020). TAp73 is activated by genotoxic stress and can induce apoptosis even in the absence of functional p53 [250, 254, 256]. It has recently been shown in p53-null human leukemia cells that expression of TAp73 mRNA can be induced by nitric oxide (NO). It was speculated that DNA damage caused by NO triggered TAp73 up-regulation [355]. Tobacco smokers are exposed to high concentrations of inhaled NO from smoke and also to endogenously released NO after uptake of nicotine into the brain [356]. Tobacco smoke is also known to contain other chemicals that have been linked to bladder tumorigenesis, mainly amines [18]. Therefore, we presume TAp73 is up-regulated in smokers due to extensive DNA

damage and/or irritation of the bladder wall not only by NO but also by potential carcinogens in tobacco smoke. TAp73 may have a protective effect on the bladder by mediating DNA damage repair or apoptosis if not inhibited by ΔNp63 or ΔNp73 isoforms or other negative regulators.

In our samples, ΔNp73 expression was tumor-specific in 7 tissue pairs, whereas in 4 tissue pairs ΔNp73 was detected only in normal but not in tumor samples. Overall, ΔNp73 mRNA was not detected in 3 tissue pairs. Loss of heterozygosity (LOH) in 1p36, where *TP73* is located, has frequently been reported in neuroblastomas and other nervous system tumors [159, 185, 186, 189, 194], in breast cancers [196, 221-223], in hepatocellular [201, 202, 224] and ovarian carcinomas [211, 226-228], but not in bladder tumors. Apart from that, *TP73* mutations that are required to completely inactivate the remaining allele have rarely been detected in bladder malignancies [219, 234]. Thus, LOH or allelic loss followed by mutation could not account for ΔNp73 loss of expression in our samples, more so when TAp73 expression is maintained. After searching in the literature for the potential role of microRNAs or acetylation processes in the regulation of *TP73* transcription, we only found that TAp73 is regulated by HDAC1 at the post-translational and not the transcriptional level [357]. Therefore, we hypothesize that transcriptional silencing of ΔNp73 in our samples could be attributed to P2 promoter hypermethylation. Aberrant methylation of the P1 *TP73* promoter was reported by Corn et al in breast cancer, colon cancer and leukemia cell lines, in acute lymphoblastic leukemias and Burkitt's lymphomas, but not in normal lymphocytes or other tissues [358]. Liu et al identified hypermethylation of the P1 *TP73* promoter in 6 NSCLC cell lines exhibiting loss of p73 mRNA and protein expression [328], whereas Daskalos et al reported frequent ΔNp73 mRNA overexpression associated with P2 promoter hypomethylation in an analysis of 101 primary NSCLCs [359]. *TP73* P1 promoter methylation was found in 7% of the squamous cell carcinomas analyzed by Gutiérrez et al, in the only study so far in bladder cancers [360]. More studies on larger patient cohorts and on transitional cell carcinomas of the bladder, which comprise the majority of the tumors, are required to define the incidence of hyper- or hypomethylation in the P1 and P2 promoters of the *TP73* gene.

We also identified TAp73 and ΔNp73 co-expression in normal bladder tissues, which could suggest overlapping functions of these isoforms. Interestingly, ΔNp73 is positively regulated by both TAp73 and wild-type p53 [262]. In malignant urothelium, TAp73 negatively correlated with ΔNp63, suggesting differential transcriptional regulation, as well as divergent functions of these two isoforms in bladder tumors.

Moreover, the ΔN/TA isoform ratio shifted 2.0-fold in favor of ΔNp73, from 0.22 in normal samples to 0.44 in tumor specimens, further indicating up-regulation of both p73 isoforms in bladder tumors.

p73 isoforms up-regulation has been detected in various types of human tumors. compared to their normal counterparts. Markedly increased TAp73 and ΔNp73 mRNA levels were measured by Zaika et al in endometrial, cervical, ovarian, vulvar and breast cancers [320]. Concin et al found ΔN´p73 overexpression in 95/100 ovarian carcinomas. TAp73 down-regulation was noted in 1/3 of the tumors and p53 mutations correlated with increased expression of N-terminally truncated p73 variants [321]. The same group later reported a strong association of ΔNp73 and ΔN´p73 overexpression with chemotherapeutic failure and with worse recurrence-free and overall survival in patients with p53 mutant cancers. In 19 cases, eight p53 mutations were found, which could functionally inhibit TAp73. These patients had a significantly shorter overall survival than those with p53 mutations of unknown effect on TAp73 [361].

ΔNp73 and TAp73 were also significantly overexpressed in a set of 117 cervical SCCs. TAp73 expression was observed in tumors sensitive to irradiation and was independently associated with better survival, whereas ΔNp73 was associated with disease recurrence and adverse patent outcome [322].

Domínguez et al analyzed p73 isoforms expression in 60 breast cancers and 113 colorectal carcinomas. Overexpression of the ex2p73 variant correlated with vascular invasion in breast cancers and with the presence of polyps in colorectal cancer patients. In the latter group, mRNA levels of ex2/3p73 were significantly higher in stage IV patients and correlated with lymph node metastasis. ΔNp73 levels also increased in parallel with disease stage and were associated with vascular invasion

[278]. These findings were confirmed by Soldevilla et al, who also identified a correlation of ex2/3p73 overexpression with reduced 5-year overall survival rate [326]. In addition, Wager et al reported that ex2/3p73 expression is a significant prognostic marker of overall survival in low-grade glioma patients, while in their study ex2p73 up-regulation correlated with worse patient outcome [316].

ΔNp73 expression has been established as an independent predictor of poor disease outcome in neuroblastoma. Analysis of 52 tumors revealed that ΔNp73 expression was confined to the tumor section with no apoptotic stain, while it significantly correlated with advanced stage and reduced overall and progression-free survival [314]. ΔNp73 overexpression has been associated with reduced survival in hepatocellular carcinoma patients [325], which are reported to express markedly elevated levels of the N-terminally truncated p73 variants [323, 324].

Moreover, significantly higher TAp73 and ΔNp73 mRNA levels have been detected in B-chronic lymphocytic leukemias [319] and prostate carcinomas [329]. Guan et al identified a significant correlation of elevated ΔNp73 expression with Gleason score of the tumors and also detected significantly higher ΔNp73 expression in patients with wild-type p53 [329]. TAp73 and ΔNp73 were up-regulated in thyroid tumors and thyroid cancer cell lines examined by Frasca et al. Yet, TAp73 was unable to induce apoptosis in thyroid cancer cells, due to its inhibition by ΔNp73 or mutant p53 [362].

It has long been established that TAp73 is activated in response to DNA damage, in some cases by mutant p53 itself, and is capable of promoting apoptosis [253, 255]. Further studies also support an anti-proliferative and anti-tumorigenic role for TAp73. It was recently demonstrated that mice lacking TAp73 but not ΔNp73 or p53, exhibited increased cell death and DNA damage in spermatogonia, malformed spermatids and marked hyperspermia. TAp73 was found to regulate expression of genes crucial for spermatid maturation and steroidogenesis, thus ensuring maintenance of mitotic cells and normal spermiogenesis [363]. In addition, TAp73β isoforms were shown to inhibit migration and invasion of cervical carcinoma cells. TAp73β but not TAp73α induced $p57^{Kip2}$ expression, thereby inhibiting the actin cytoskeleton dynamics and cancer cell motility. On the contrary, TAp73α exhibited a positive effect on cell migration and

invasion in these cells [364]. TAp73 is also known to induce apoptosis after cisplatin treatment in breast cancer cells [345]. In ovarian cancer cells treated with cisplatin, TAp73 was identified as an activator of the JNK apoptotic pathway via up-regulation of GADD45α and MKK4 proteins. Thus, it was shown that, apart from transcriptionally activating pro-apoptotic genes like *Bax*, *NOXA* and *PUMA*, TAp73 can use other mechanisms as well to induce apoptosis in response to chemotherapy [365]. Interestingly, p53 isoforms can have diverse effects on TAp73 transcriptional activity and apoptosis. TAp73α was found to form complexes only with Δ133p53α/β/γ isoforms, whereas all Δ40p53 and Δ133p53 isoforms could form complexes with TAp73β in p53-null human lung cancer cells. All p53 isoforms were found to counteract TAp73β transactivation function. TAp73β apoptotic activity was augmented by co-expression with p53β and inhibited by Δ133p53α/β isoforms [366].

In contrast to TAp73, which has tumor-suppressive functions, multiple lines of evidence indicate that ΔNp73 can promote oncogenesis and tumor aggressiveness. ΔNp73 is capable of enhancing TGF-β promoter activity in hepatocarcinoma cells, resulting in expression of genes involved in tumor invasion and metastasis [261]. In melanoma cells, ΔNp73 has been shown to facilitate and EMT-like phenotype by down-regulating E-cadherin and up-regulating Slug. ΔNp73 expression in tumor xenografts enhanced their ability to invade and metastasize, without affecting tumor growth [367]. ΔNp73 was shown to promote proliferation of thyroid cancer cells. Increased ΔNp73 mRNA levels reduced levels of PTEN, which is a molecule involved in attenuation of tyrosine kinase signaling. ΔNp73 also increased phosphor-Akt protein content and Mdm2-mediated p53 degradation, resulting in enhanced cell proliferation and reduced apoptotic rates [368]. ΔNp73 can also inhibit apoptosis by mechanisms that do not involve physical interaction or competition with TAp73 or wild-type p53. It was demonstrated in hepatocellular carcinoma cells that ΔNp73 can negatively regulate the death receptor genes *CD95*, *TNF-R1*, *TRAIL-R2* and *TNFRSF18*. ΔNp73 can also repress genes encoding caspase -2, -3, -8 and -9. Overall, it was shown that ΔNp73 can block both the intrinsic and extrinsic apoptotic pathways in hepatocellular carcinoma cell lines [369].

To the best our knowledge, this is the first study to measure transcriptional levels of all p53 family members and of p63 and p73 TA and ΔN isoforms simultaneously in bladder tumors and normal tissues and to correlate these with patients' clinicopathological parameters. The results we obtained are mostly in concurrence with the ones emerging from previous reports in urothelial carcinomas and other human malignancies. In our study, p63 overexpression was associated with a less aggressive tumor phenotype, whereas p73 up-regulation correlated with an unfavorable patient outcome. We also provide evidence that p63 and p73 exert their contribution to the malignant transformation of the bladder epithelium independently of p53. In addition, our results support an anti-invasive role for ΔNp63 in urothelial cancers and a potentially protective effect of TAp73 on bladder epithelium. Finally, our results provide evidence for epigenetic regulation of p63 and p73 isoforms expression in normal and malignant urothelium.

Despite the mounting evidence implicating all p53 family members in human neoplasia, many questions have yet to be answered concerning the exact role of these genes in bladder tumorigenesis and disease progression. More detailed studies, employing larger patient cohorts, as well as functional studies in cell lines, are required in order to assess the contribution of p53 and its homologues in urinary bladder malignancies.

Over the years of intense research on the p53 family of transcription factors, it is becoming all the more clear that we are dealing with genes encoding proteins with oncogenic or tumor-suppressive potential, rather than classic oncogenes or tumor suppressors. The need for rapid and sensitive detection of all p53 family protein isoforms, as well as the assessment of their diagnostic or predictive value in the clinical setting, remains the greatest challenge to be met in the near future, yet appears very promising as far as effective patient management is concerned.

APPENDIX

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Α. ΑΝΑΡΤΗΜΕΝΕΣ ΑΝΑΚΟΙΝΩΣΕΙΣ ΣΕ ΕΛΛΗΝΙΚΑ ΣΥΝΕΔΡΙΑ

1. Ε. Βακωνάκη, Ν. Σουλιτζής, Σ. Σηφάκης, **Δ. Παπαδογιάννη** και Δ.Α. Σπαντίδος. Μελέτη της έκφρασης και της μεθυλίωσης των αμινοτελικών ισομορφών της πρωτεΐνης p73 στον καρκίνο του ενδομητρίου. 12^{ο} Πανελλήνιο Συνέδριο στη Μαιευτική και Γυναικολογία, 17-20 Μαΐου 2012, Θεσσαλονίκη.

2. Ε. Βακωνάκη, Ν. Σουλιτζής, Σ. Σηφάκης, **Δ. Παπαδογιάννη**, Δ. Κουτρουλάκης και Δ.Α. Σπαντίδος. Υπερέκφραση και μεταβολή της αναλογίας των ισομορφών ΔNp63 και TAp63 στον καρκίνο του ενδομητρίου: συσχέτιση με παχυσαρκία, εμμηνόπαυση και βαθμό διαφοροποίησης. 12^{ο} Πανελλήνιο Συνέδριο στη Μαιευτική και Γυναικολογία, 17-20 Μαΐου 2012, Θεσσαλονίκη.

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1. **D. Papadogianni**, N. Soulitzis, D. Delakas and D.A. Spandidos. Expression analysis of TAp63 and TAp73 isoforms in bladder cancer. 13th World Congress on Advances in Oncology and 11th International Symposium on Molecular Medicine, 9-11 October 2008, Hersonissos, Crete, Greece. International Journal of Molecular Medicine, 22 (Supplement 1): S90, 2008.

2. G. Panagiotakis, **D. Papadogianni**, N. Soulitzis, G. Sourvinos and D.A. Spandidos. Absence of human papilloma virus infection in normal and malignant bladder. 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine, 15-17 October 2009, Loutraki, Greece. International Journal of Molecular Medicine, 24 (Supplement 1): S80, 2009.

3. G. Panagiotakis, **D. Papadogianni**, N. Soulitzis, G. Sourvinos and D.A. Spandidos. High prevalence of human Herpesviruses in urothelial carcinomas. 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine, 15-17 October 2009, Loutraki, Greece. International Journal of Molecular Medicine, 24 (Supplement 1): S80, 2009.

4. **D. Papadogianni**, N. Soulitzis and D.A. Spandidos. mRNA expression of ΔNp63 and ΔNp73 isoforms in urothelial malignancies. 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine, 15-17 October 2009, Loutraki, Greece. International Journal of Molecular Medicine, 24 (Supplement 1): S81, 2009.

5. **D. Papadogianni**, N. Soulitzis and D.A. Spandidos. The role of p53 gene family in bladder cancer. 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine, 15-17 October 2009, Loutraki, Greece. International Journal of Molecular Medicine, 24 (Supplement 1): S81, 2009.

6. E. Vakonaki, N. Soulitzis, **D. Papadogianni**, S. Sifakis and D.A. Spandidos. Methylation and expression profile of p73 in endometrial carcinoma. 15th World Congress on Advances in Oncology and 13th International Symposium on Molecular Medicine, 7-9 October 2010, Loutraki, Greece. International Journal of Molecular Medicine, 26 (Supplement 1): S95, 2010.

7. E. Vakonaki, N. Soulitzis, S. Sifakis, **D. Papadogianni**, D. Koutroulakis and D.A. Spandidos. Overexpression and ratio disruption of ΔNp63 and TAp63 isoforms in endometrial adenocarcinoma. 16th World Congress on Advances in Oncology and 14th International Symposium on Molecular Medicine, 6-8 October 2011, Rhodes, Greece. International Journal of Molecular Medicine, 28 (Supplement 1): S75, 2011.

ΣΥΜΜΕΤΟΧΗ ΣΕ ΟΡΓΑΝΩΣΗ ΕΠΙΣΤΗΜΟΝΙΚΩΝ ΣΥΝΕΔΡΙΩΝ

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ΔΗΜΟΣΙΕΥΣΕΙΣ (ΞΕΝΟΓΛΩΣΣΕΣ)

1. Vakonaki E, Soulitzis N, Sifakis S, **Papadogianni D**, Koutroulakis D, Spandidos DA. Overexpression and ratio disruption of ΔNp63 and TAp63 isoform equilibrium in endometrial adenocarcinoma: correlation with obesity, menopause, and grade I/II tumors. J Cancer Res Clin Oncol. 2012 Aug;138(8):1271-8.

2. Panagiotakis GI, **Papadogianni D**, Chatziioannou MN, Lasithiotaki I, Delakas D, Spandidos DA. Association of human herpes, papilloma and polyoma virus families with bladder cancer. Tumour Biol. 2013 Feb;34(1):71-9. ***(equal contribution)***

3. **Papadogianni D**, Soulitzis N, Delakas D, Spandidos DA. Expression of p53 family genes in urinary bladder cancer: correlation with disease aggressiveness and recurrence. Tumour Biol. 2014 Mar;35(3):2481-9.

ΔΗΜΟΣΙΕΥΣΕΙΣ ΥΠΟ ΣΥΓΓΡΑΦΗ

1. **D. Papadogianni**, N. Soulitzis, D. Delakas and D.A. Spandidos. Expression analysis of TA and ΔN p63 and p73 isoforms in bladder cancer.
2. **D. Papadogianni**, N. Soulitzis and D.A. Spandidos. The TP53 family members in urinary bladder cancer.

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RESEARCH ARTICLE

Expression of p53 family genes in urinary bladder cancer: correlation with disease aggressiveness and recurrence

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Abstract p53 is a tumour suppressor gene with an established role in the majority of human neoplasias. Its homologues—p63 and p73—cannot be classified as tumour suppressors, since they encode isoforms with oncogenic properties as well. p63 plays a crucial role in epithelial cell differentiation and p73 is essential for neuronal cell development. The p63 and p73 expressions have been investigated in a variety of human tumours including bladder carcinomas; yet, this is the first study to simultaneously analyse the transcriptional levels of all p53 family members in bladder cancer. Using quantitative real-time polymerase chain reaction, we measured the mRNA expression of p53, p63 and p73 in 30 bladder tumours, each paired with adjacent normal tissue. All three studied genes were up-regulated in malignant specimens, p53 by 1.9-fold, p63 by threefold and p73 by twofold, respectively. Further analysis suggested that p63 and p73 act independently of p53 in the malignant bladder epithelium. Statistical analysis revealed that p63 overexpression was more frequent in recurrent bladder tumours ($p = 0.045$) and in older patients ($p = 0.022$). Papillary tumours also exhibited abnormal p63 expression ($p = 0.026$). Finally, p73 was up-regulated in Grade III one-site tumours ($p = 0.040$). Our results indicate that all p53 family members are abnormally expressed in bladder cancer but do not act synergistically. High levels of p63 correlate with non-muscle invasive tumours with frequent relapses, whereas p73 overexpression is associated with a more aggressive tumour phenotype.

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Keywords p63 · p73 · Real-time PCR · mRNA · Bladder cancer · Biomarkers

Introduction

Urinary bladder cancer is the 11th most common malignancy worldwide [1] and is more common in developed countries, with an estimated 72,570 new cases and 15,210 cancer-related deaths for 2013 in the USA alone [2]. Tobacco use and occupational exposure to aromatic amines are recognised as major risk factors for the development of bladder transitional cell carcinoma (TCC), whereas *Schistosoma* infection in certain geographical regions accounts for most bladder squamous cell carcinomas (SCCs) [1]. Chronic urinary tract infection, exposure to cyclophosphamide and radiotherapy to adjacent organs are also known to contribute to the development of the disease [3].

Genetic alterations underlying the development of bladder cancer include chromosomal aberrations, inactivating tumour-suppressor gene mutations and activation of oncogenes [4]. The two major phenotypic variants of the disease, low-grade non-muscle-invasive and high-grade muscle-invasive tumours, arise via two mutually exclusive genetic events. FGFR3, a tyrosine kinase receptor involved in cell proliferation and differentiation, is mutated in more than 70 % of non-muscle-invasive tumours [5], and these mutations have been associated with good prognosis for patients presenting with low-grade tumours [6]. On the contrary, p53 is mutated in almost 50 % of muscle-invasive lesions [7], resulting in diverse effects on downstream anti-apoptotic molecules and providing a marker for poor prognosis and unfavourable response to therapy [8].

p63 and p73 are recently identified p53 homologues. Both genes have physiological roles in different types of cells; p63

is indispensable for normal epithelial development [9], whereas p73 is required for proper neurogenesis [10]. p53 mutational inactivation is believed to be one of the primary events triggering malignant transformation and is detected in almost 50 % of cancers [11]; however, p63 and p73 mutations are infrequently detected in human tumours [12]. All family genes use alternative splicing and alternative promoters to generate multiple N- and/or C-terminally truncated protein isoforms; p53 also uses alternative translation initiation sites [13–15].

Aberrant p53 expression has been reported in SCCs, hematological and gynecological malignancies [15], as well as in bladder TCCs [16]. p63 is involved to a greater extent in SCCs, especially of the head and neck, by increasing the invasive potential of tumour cells [17] and its overexpression is associated with an adverse disease outcome [18]. On the contrary, in TCCs of the urinary bladder, the decrease or loss of p63 expression has been associated with disease progression and poor prognosis [16, 19]. p73 overexpression has also been detected in SCCs, as well as in hematological malignancies [20], and its overexpression in bladder carcinomas has been associated with advanced tumour stage and grade [21].

In the current study, p53, p63 and p73 mRNA expression was analysed in a set of 30 urinary bladder tumours, matched with normal tissues, in order to determine the expression levels of p53 family genes in bladder cancer and to evaluate their association with the development and progression of bladder malignancies.

Methods

Study population

Thirty TCCs were collected from patients diagnosed with urinary bladder cancer who underwent cystectomy at the Department of Urology, Asklipeiion Hospital, Voula, Athens, Greece. Each tumour sample was matched to a non-cancerous specimen derived from normal adjacent bladder tissue. Following surgical resection, all tissue samples were snap-frozen at −80 °C until use. Mean age at cystectomy was 72.2±10.6 years (range 44–86). All tumour specimens were histopathologically examined using hematoxylin- and eosin-stained slides in order to determine tumour stage and grade. Normal adjacent tissue samples had no contaminating tumour cells as confirmed by the histopathological examination. The clinicopathological parameters of the study population are listed in Table 1. The Ethics Committee of the Medical School of the University of Crete approved the current study and written informed consent was obtained from all the patients or their relatives.

Table 1 Patients' clinicopathological characteristics

Characteristic	Bladder cancer (%)
Cases (*n*)	30
Age (mean±SD, years)	72.2±10.6
Gender	
Male	27 (90.0)
Female	3 (10.0)
Tumor stage	
Ta–T1	18 (60.0)
T2–T4	12 (40.0)
Tumour grade	
II	10 (33.3)
III	20 (66.7)
Tumour type	
Papillary	23 (76.7)
Nonpapillary	7 (23.3)
Recurrence	
Primary	24 (80.0)
Recurrent	6 (20.0)
Tumour sites	
One	22 (73.3)
Multiple	8 (26.7)
Therapy[a]	
Yes	18 (64.3)
No	10 (35.7)
Relapse[a]	
Yes	7 (36.8)
No	12 (63.2)
Survival	
Yes	22 (73.3)
No	8 (26.7)
Smoking habit	
Current smokers	18 (60.0)
Ex-smokers	8 (26.7)
Non-smokers	4 (13.3)
Occupational exposure[b]	
Yes	19 (63.3)
No	11 (36.7)
Family cancer history[a]	
Yes	9 (33.3)
No	18 (66.7)

[a] Data missing from some cases

[b] To chemicals, paints, pesticides, petroleum, ink, etc

RNA extraction and cDNA synthesis

Tissue specimens were homogenised in TRI Reagent® (Ambion, Austin, TX) using a power homogeniser. Following tissue homogenisation, samples were centrifuged in order to

remove insoluble material (fat, polysaccharides) and high molecular weight DNA from the homogenates. The supernatants were transferred to fresh tubes and incubated at room temperature, followed by chloroform addition and centrifugation. Total RNA was precipitated from the supernatant with isopropanol, washed with 75 % ethanol, resuspended in 50 μl RNase-free water and stored at −80 °C until future use. RNA concentration and purity were determined by measuring its 260 nm absorbance and its 260/280 nm absorbance ratio, respectively, on a UV spectrophotometer.

cDNA was synthesised by reverse transcription (RT) with the RETROscript® Kit (Ambion), using the two-step RT-PCR protocol. In detail, 2 μg of total RNA, 8.3 μM of random decamers and nuclease-free water up to a final volume of 12 μl were incubated for 3 min at 85 °C for the heat denaturation of RNA secondary structures. Samples were immediately placed on ice for the addition of the remaining RT mix, which contained 10× RT Buffer (500 mM Tris–HCl, pH 8.3, 750 mM KCl, 30 mM $MgCl_2$, 50 mM DTT), dNTP mix (2.5 mM each dNTP), 10 units RNase inhibitor and 100 units MMLV-RT. The final mix (total volume 20 μl) was incubated at 44 °C for 60 min for the RT reaction to take place, followed by incubation at 92 °C for 10 min to inactivate the reverse transcriptase. cDNAs were stored at −20 °C until future use.

Real-time PCR

mRNA expression levels of the three p53 family genes were measured using a real-time RT-PCR assay with SYBR® Green I. Beta-Actin (β-Actin) was used as an internal control in order to normalise p53, p63 and p73 mRNA expression levels. The mRNA-specific primers used are listed in Table 2. All primer pairs were designed to simultaneously detect both wild-type/full-length and mutant/truncated transcripts of the p53 family genes and span at least one intron > 800 bp in order to avoid co-amplification of contaminating genomic DNA.

Each reaction was performed in a final volume of 20 μl containing 1 μl cDNA from tumour or normal samples, 2× Maxima™ SYBR Green quantitative real-time polymerase chain reaction (qPCR) Master Mix (Fermentas Life Sciences, Vilnius, Latvia) and 100 nM of each primer pair. After initial denaturation at 95 °C for 10 min, PCR was performed for 40 cycles comprising of denaturation at 95 °C for 30 s, annealing at 60 °C (for p53 and β-Actin) or 62 °C (for p63 and p73) for 30 s and elongation at 72 °C for 30 s, followed by a melt-curve analysis, in which the temperature was increased from 60 to 95 °C at a linear rate of 0.2 °C/s. Data collection was performed both during annealing and extension, with two measurements at each step, and at all times during melt-curve analysis. A representative pool of all cDNA samples was diluted in a series of six twofold dilutions and was run in the same plate in order to construct a standard curve for the quantification process. In each reaction, two non-template negative controls were included. All PCR experiments were performed on the Mx3000P real-time PCR thermocycler using the software version 4.1 (Stratagene, La Jolla, CA). For all samples, excluding those with no amplification plots or with dissociation curves indicating primer–dimer accumulation or by-product formation, the expression of genes of interest was calculated as follows: the Ct values of all unknown samples were projected, with the help of the standard curve, to a relative mRNA quantity. This value was then divided by the same sample's β-actin mRNA value, for normalisation purposes. The normalised values of each malignant bladder samples were then divided by the normalised values of their adjacent normal samples. A twofold increase (a value≥2) or decrease (a value≤0.5) in expression was considered biologically significant (overexpression or down-regulation, respectively).

Ten microliters of PCR products were resolved on 2 % (w/v) agarose gels, stained with ethidium bromide and photographed on a UV light transilluminator, in order to verify the results of the melt-curve analysis.

Statistical analysis

p53, p63 and p73 mRNA levels were first evaluated by one-sample Kolmogorov–Smirnov goodness of fit test, in order to determine whether they follow a normal distribution pattern. Depending on the results, Pearson's correlation or the non-

Table 2 Primer sequences, PCR annealing temperatures and amplicon sizes

Primer pair	Sequence (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)
p53	GTG AGC GCT TCG AGA TGT TC ATG GCG GGA GGT AGA CTG AC	60	137
p63	CCT CCA ACA CCG ACT ACC CAG GCA CCG CTT CAC CAC TCC	62	236
p73	GAA ACG CTG CCC CAA CCA C CAC CAC GAC GCT CTG CCT G	62	142
β-actin	CGG CAT CGT CAC CAA CTG GGC ACA CGC AGC TCA TTG	60	70

parametric Spearman rank correlation was used in order to determine their relation pair-wise and their correlation with continuous variables (age). Moreover, their association with categorical variables (tumour stage, family cancer history, etc.) was determined by Student's t test (after examining for equality of variances with Levene's test) or by its nonparametric equivalents Mann–Whitney U and Kruskal–Wallis H tests. Finally, the χ^2 test, using Fisher's exact test when indicated by the analysis, was used to examine p53, p63 and p73 expression status in association with the various clinicopathological parameters after stratification. All statistical analyses were two-sided and performed using SPSS 11.5 software (SPSS, Chicago, IL). Statistical significance was set at the 95 % level (p value<0.05).

Results

In the present study, the mRNA expression profile of the p53 gene family (p53, p63 and p73) was examined using a qPCR assay in normal and malignant samples from 30 patients diagnosed with bladder cancer.

Mean age at diagnosis was 72.2 years, with the majority of patients (90 %) being males. Histological examination revealed that at the time of diagnosis 60 % of the malignant lesions were non-muscle-invasive (stages Ta–T1), whereas 40 % were muscle-invasive (stages T2–T4). Additionally, 80 % of the patients presented with primary tumours, whilst 20 % had recurrent disease. Statistical analysis revealed that the majority of non-muscle-invasive tumours were moderately differentiated (Grade II) papillary lesions, whereas most muscle-invasive tumours were poorly differentiated (Grade III), non-papillary lesions (p= 0.002 and p= 0.009, respectively). Additionally, disease recurrence was associated with multiplicity of tumours in the bladder wall (p= 0.007). Finally, as expected, non-muscle-invasive carcinomas were associated with a favourable clinical outcome, whereas patients with muscle-invasive lesions, although treated with radical cystectomy, succumbed to the disease (p= 0.003).

p53 was overexpressed in 14/30 (47 %) samples, down-regulated in 3/30 (10 %), whilst in 13/30 (43 %) specimens its expression was normal. p63 and p73 expression pattern was slightly different. p63 and p73 were overexpressed in 12/30 (40 %) and 13/30 (43 %) specimens, respectively, whilst they were down-regulated in 8/30 (27 %) and 7/30 (23 %) samples, respectively. For both genes, 33 % of samples (10/30) had normal expression (Fig. 1).

Further analysis showed that p53 exhibited a 1.9-fold overexpression in malignant specimens (from 0.99±0.13 in normal samples to 1.89±0.25 in tumour samples). Similarly, p63 was overexpressed by threefold in malignant specimens (from 1.04±0.26 in normal samples to 3.09±0.86 in tumour samples). Finally, p73 was overexpressed by twofold in malignant specimens (from 0.58±0.10 in normal samples to 1.19±0.21 in tumour samples; Fig. 2).

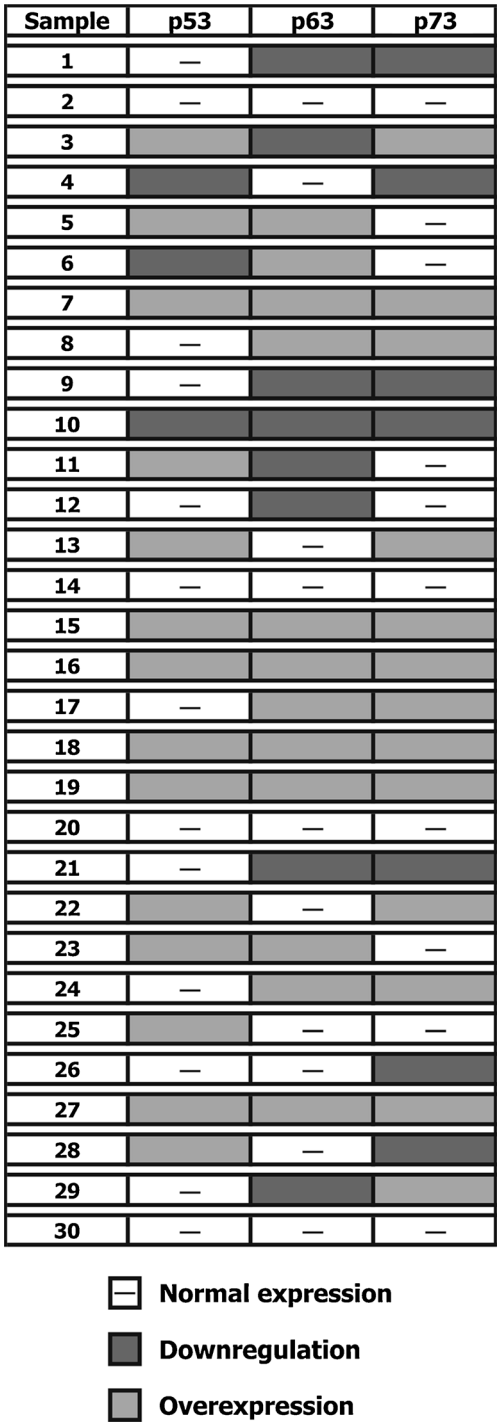

Fig. 1 Schematic representation of p53, p63 and p73 expression profile in our series of urinary bladder cancer tissue samples

With the Spearman rank correlation, we tested the co-expression patterns of the three examined genes in a pair-wise manner in the normal and malignant bladder epithelium. This test examines whether two genes are up-regulated or down-regulated together (positive correlation), or whether

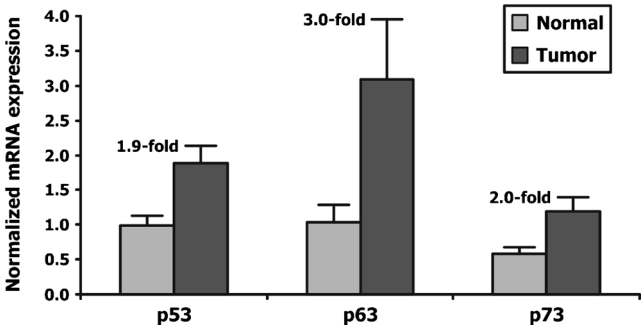

Fig. 2 Bar chart depicting p53, p63 and p73 normalised expression in normal and malignant bladder specimens, respectively. Floating numbers represent fold change between the two sample groups

when one gene is overexpressed and the other has a reduced expression (negative correlation). Results are displayed in Table 3. In the normal bladder epithelium, all members of the p53 gene family were co-expressed together. However, in the malignant bladder epithelium, only the positive co-expression between p63 and p73 was maintained, whilst p53 was no longer co-expressed with either p63 or p73.

Statistical analysis revealed that p53 was up-regulated in non-muscle-invasive tumours compared with muscle-invasive ones (2.34±0.36 vs. 1.21±0.19, p= 0.047). Moreover, significantly higher p63 mRNA levels were measured in malignant samples from patients > 75 years of age versus younger patients (4.56±1.61 vs. 1.63±0.59, p= 0.022) and from patients with disease relapse compared with cancer-free patients (8.22±4.18 vs. 2.02±0.49, p= 0.045). In addition, p63 expression deviated from the normal pattern in papillary versus non-papillary lesions (3.61±1.16 vs. 1.55±0.53, p= 0.026). Finally, p73 overexpression was observed in Grade III tumours compared with Grade II tumours, in patients presenting with one-site lesions (1.22±0.28 vs. 0.70±0.26, p= 0.040; Fig. 3).

Table 3 p53, p63 and p73 pair-wise co-expression analysis in normal and malignant bladder epithelium

		p53	p63	p73
Normal bladder epithelium				
p53	CC	1.000		
	P value			
p63	CC	0.640	1.000	
	P value	**<0.001**		
p73	CC	0.715	0.641	1.000
	P value	**<0.001**	**<0.001**	
Malignant bladder epithelium				
p53	CC	1.000		
	P value			
p63	CC	0.221	1.000	
	P value	0.26		
p73	CC	0.290	0.493	1.000
	P value	0.12	**0.008**	

CC Correlation coefficient

Discussion

p53 is the most extensively studied tumour-suppressor gene, known to play a key role in sequestering cellular messages and subsequently mediating cell cycle arrest or programmed cell death [15, 22] (Fig. 4). p53 mutational activation has a significant contribution to the development and progression of human tumours.

The recent recognition of p53 homologues lead to the belief that p63 and p73 are p53 co-players in the tumour suppression field. However, both p63 and p73, although highly similar with p53 regarding their genomic sequence [23], are not classic Knudson-type tumour-suppressor genes. Unlike p53, p63 and p73 mutations are infrequent in human cancers [12], whereas their deletion results in developmental and neurological abnormalities rather than the formation of malignant lesions [9, 10]. Differential transcription via a cryptic promoter and alternative splicing account for the generation of a large number of N- and C-terminal isoforms, respectively, of both p63 and p73. Full-length TAp63 and TAp73 share similar transactivating properties with p53 [13, 14] and are hence believed to promote apoptosis. On the contrary, ΔNp63 and ΔNp73, which lack the N-terminus, contribute to malignant transformation via the inhibition of p53 and their respective TA isoforms in a dominant-negative manner [12] (Fig. 4).

In our study, p53 mRNA levels were elevated in 47 % of malignant specimens, with this up-regulation being more frequent in non-muscle-invasive bladder carcinomas than in muscle-invasive ones (p= 0.047). However, this finding is in contrast with the results obtained in a large cohort study of 243 bladder cancer patients, where p53 nuclear positivity was identified as a prognostic factor of disease progression, independently of tumour stage and grade [24]. Similar results were obtained by Zigeuner et al. [16], who reported an association of p53 overexpression in advanced stage and grade bladder tumours, along with no p53 immunoreactivity in normal bladder and renal tissues. Mutant p53 protein has a prolonged half-life when compared with the wild-type one and is detected in high levels in cancerous cells [24], where it facilitates the angiogenic potential of the tumours [12, 25]. Of interest, George et al. [26] recently reported that mutations in specific exons of the p53 gene do not result in p53 functional inactivation and may be associated with a more favourable patient outcome, at least in terms of disease recurrence. Moreover, it has been shown that p53 up-regulation can be attributed to other aberrations as well, such as mdm2

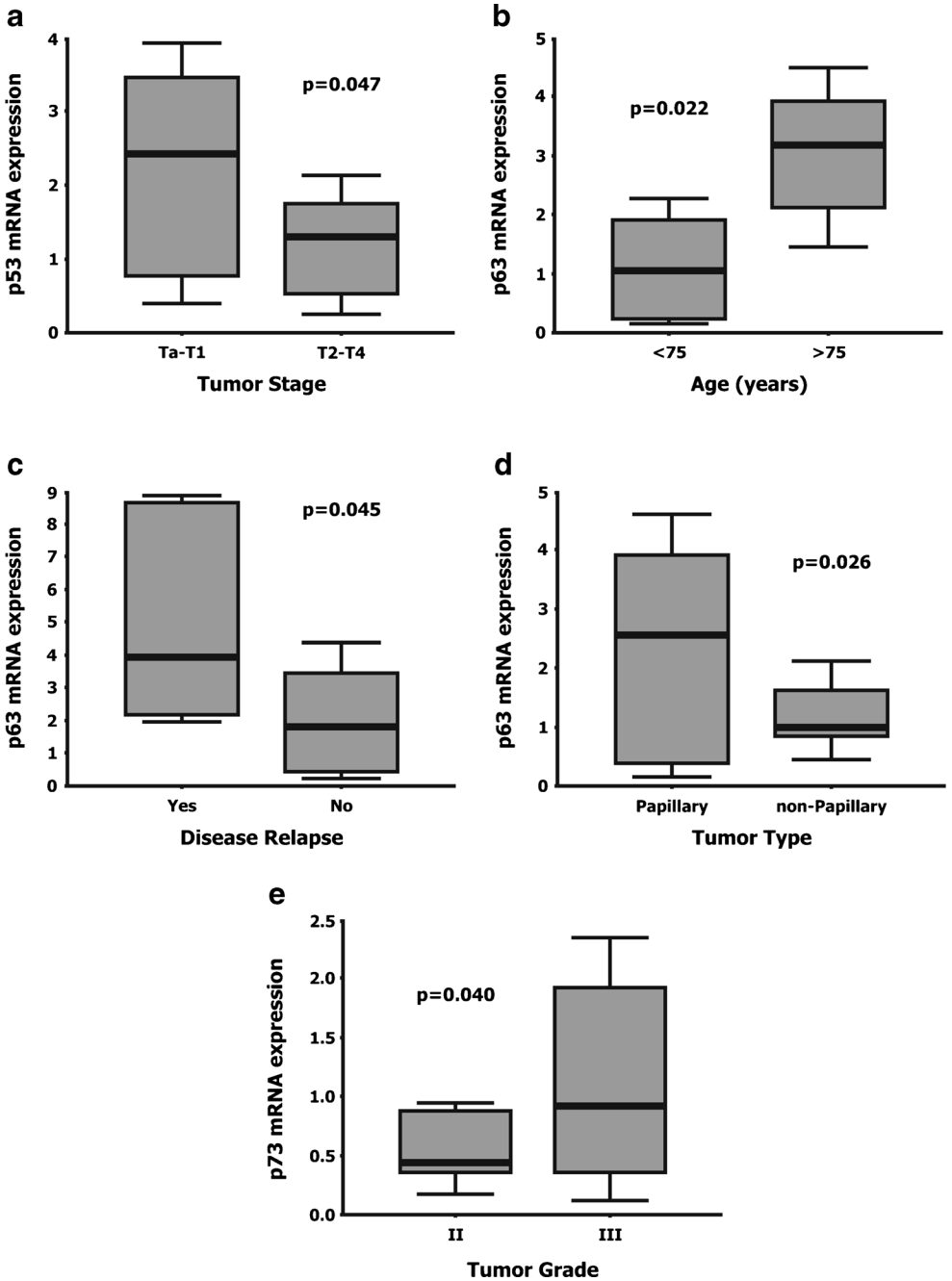

Fig. 3 Box and whisker plots depicting statistically significant associations. **A** p53 was up-regulated in non-muscle-invasive tumours compared with muscle-invasive ones ($p = 0.047$). **B** Significantly higher p63 mRNA levels were measured in malignant bladder samples from patients > 75 years of age versus younger patients ($p = 0.022$) and **C** from patients with disease relapses compared with cancer-free patients ($p = 0.045$). **D** p63 expression deviated from the normal pattern in papillary versus nonpapillary lesions ($p = 0.026$). **E** p73 overexpression was observed in Grade III tumours compared with Grade II tumours, in patients presenting with one-site lesions ($p = 0.040$). The *thick line* near the center of each rectangular box represents the median value, the bottom and top edges of the box indicate the 1st (Q_1) and 3rd (Q_3) quartiles, and the ends of the whiskers depict the 10th (P_{10}) and 90th (P_{90}) percentiles

overexpression, a negative regulator of p53 ability to transactivate target genes [27]. In that context, we believe that data on the mutational status of the p53 gene and/or the expression status of other proteins participating in the p53 pathway in our set of bladder tumours would be essential to evaluate its expression profile thoroughly and to interpret its association with clinicopathological parameters.

p63 was up-regulated in 40 % of cancer samples and its overexpression was associated with late disease onset ($p = 0.022$). To our knowledge, this is the first study to date

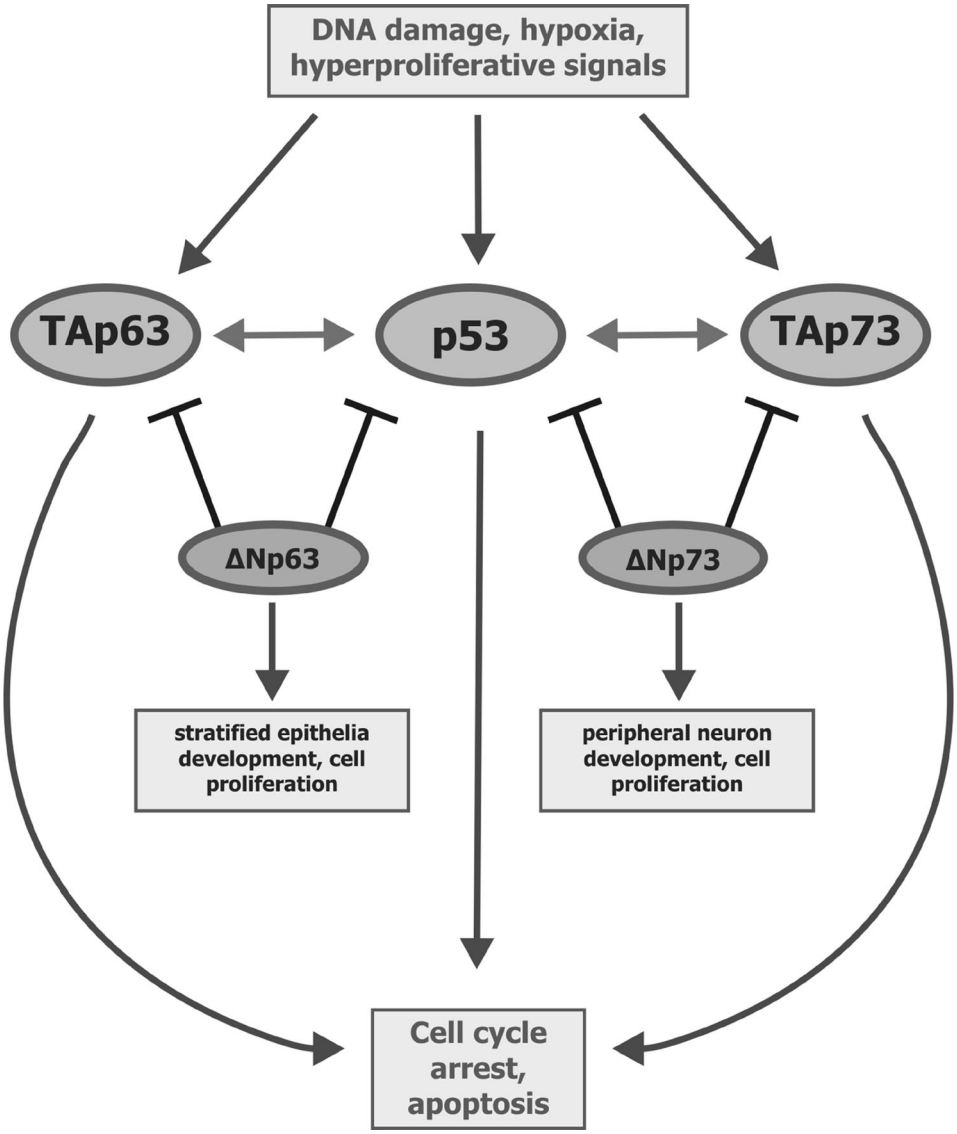

Fig. 4 Roles of p53 family members in cell cycle control and development. Full-length TA isoforms cooperate with p53 in the induction of apoptosis, via transactivation of target genes. ΔN isoforms act as dominant negative inhibitors of p53 and their respective TA isoforms; they have oncogenic potential and are also implicated in developmental pathways

to report an association of p63 expression with patient age. A meta-analysis of age-related gene expression profiles using datasets from mice, rats and humans showed that genes implicated in inflammation and immune response are overexpressed with age [28]. Yang et al. [29] found that ΔNp63 overexpression in SCC cell lines and transgenic mice resulted in increased epithelial proliferation, enhanced pro-inflammatory molecule expression and decreased expression of p21, a protein involved in cell cycle arrest. In addition, p63 was found to be significantly overexpressed in patients with recurrent bladder tumours ($p = 0.045$), which are mainly multifocal, non-muscle-invasive papillary neoplasms [4]. Furthermore, p63 aberrant expression was frequently observed in papillary tumours ($p = 0.026$), providing some first evidence on its association with bladder tumour histopathology. These results are similar to those obtained in previous studies, in which low-grade non-muscle-invasive papillary tumours were found to express p63 more frequently and in higher levels, as compared with their high-grade muscle-invasive counterparts [19, 30]. Koga et al. [30] also found that high p63 levels were associated with better prognosis.

p73 overexpression was detected in 43 % of specimens and was significantly more frequent in poorly differentiated one-site tumours ($p = 0.040$), which are often muscle-invasive and life-threatening [4]. These results are in accordance with those obtained in earlier studies [21, 31], in which it was also demonstrated that p53 mutation or altered expression does not correlate with p73 expression in bladder tumours. Unlike p63, p73 expression was frequently detected in adenocarcinomas

of the colon and breast, where its overexpression was correlated with poor patient outcome [32–34]. There is also evidence supporting that the N-terminally truncated p73 isoforms can contribute to an aggressive tumour phenotype by up-regulating the expression of genes implicated in invasiveness, apoptosis and drug resistance [33].

Wild-type p53 protein has long been designated as 'the guardian of the genome' and p53-deficient mice are known to have a strong predisposition to cancer [35]. p53 sequence alteration is a key event in the vast majority of human malignancies, yet this does not apply for its homologues. In our study, all p53 family genes were found to be co-expressed in normal bladder tissue, yet this co-expression pattern was disrupted in malignant bladder epithelium, where only p63 and p73 were co-expressed. This finding, which has also been observed by Chi et al. [21], suggests that p63 and p73 act synergistically and independently of p53 and poses questions about the transcriptional regulation of p53 and its homologues in normal and malignant bladder. Earlier studies suggest that p63 and p73 respond to different elements, including p53, in terms of their transcriptional activation in human keratinocytes, as well as in prostate and breast tumour cells [36]. This has yet to be confirmed or contradicted by respective studies in bladder cells.

It has been previously demonstrated that high transcriptional levels of p53 family genes often correlate with high protein levels. Dekairelle et al. [37] measured increased p53 mRNA levels in TCC tumour samples, in the majority of which p53 protein was also detected in high levels using immunohistochemistry. Additionally, significant correlation have been observed between ΔNp63 mRNA and protein levels both in bladder TCC and SCC cell lines [19] and in tumour specimens [30]. Finally, in a study by Puig et al. [38], high TAp73 transcriptional levels correlated with high TAp73α protein levels in bladder cancer cell lines.

To our knowledge, this is the first study to measure transcriptional levels of all p53 family members simultaneously and to correlate these with patients' clinicopathological parameters. The results we obtained are mostly in concurrence with the ones emerging from previous reports. In our study, p63 overexpression was associated with a less aggressive tumour phenotype, whereas p73 up-regulation correlated with an unfavourable patient outcome. We also provide evidence that p63 and p73 exert their contribution to the malignant transformation of the bladder epithelium independently of p53. Despite the mounting evidence implicating all p53 family members in human neoplasia, many questions have yet to be answered concerning the exact role of these transcription factors in bladder tumorigenesis. More detailed studies, employing larger patient cohorts, as well as functional studies in cell lines, are required in order to elucidate the role of p53 and its homologues in urinary bladder malignancies, along with potential interactions between the protein isoforms encoded by these genes.

Conflicts of interest None

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