Molekulare Pathologie
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E2F3 is responsible for frequent amplification of 6p22.3 in human bladder cancer

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1 INTRODUCTION

A healthy multicellular organism depends on continuous cell turnover, to replace old cells. Therefore, a proportion of cells are permanently required to divide and differentiate in order to repopulate living organs. Stem cells are responsible for roughly 10^{12} divisions per day, and even in organs with low turnover rates, massive proliferation can be initiated by events such as trauma or infection. Regardless of such an enormous production of new cells, the adult human body maintains a constant weight. Even obesity is not primarily the result of increased cell multiplicity but of increased volume and thus mass of adipocytes. In multicellular organisms, however, this exquisite coordination can only be achieved through a sophisticated network of overlapping molecular mechanisms, governing the birth and death rates of the cells.

Cancer cells ignore the most basic rules of how multicellular organisms are produced and maintained. Their behaviour characteristically involves accelerated growth, invasion, avoidance of senescence, enhanced stimulation of angiogenesis and genome instability.

Defects, that tangent to 'the balance of birth and death', have the potential to disrupt the social control, if they cannot be corrected immediately. In a human body with such a large mass of cell divisions per day, billions of cells experience molecular disturbances or mutations every day. Under particular circumstances, a mutation may give a selective advantage to a cell, allowing it to divide more frequently than its neighbours and to become a founder of a growing mutant clone (see **figure 1**).

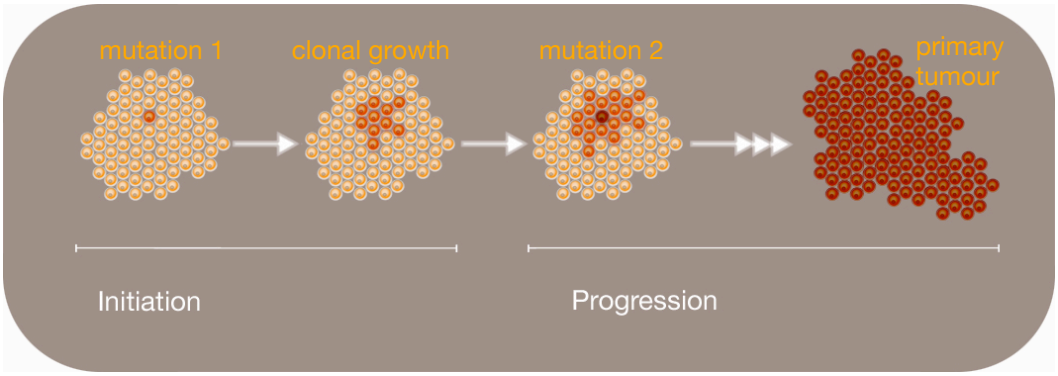

Figure 1: The succession leading to a primary tumour starts with a particular mutation in a single cell. This mutation may provide a selective advantage that allows this cell to divide more rapidly than the adjacent cells. Thereby it may become a founder of a growing mutant clone. Repeated rounds of mutation, competition, and natural selection eventually lead to a clone of fully malignant cancer cells. At each step, a specific mutation enhances cell proliferation, so that newly modified mutant clones become dominant over predecessors. Enhancing proliferation speed and population size of the developing clone additionally increases the risk for further mutations.

If the cancerous cell and its progeny remain clustered together in a single mass, the tumour is said to be benign. As soon as the tumour cells have acquired the ability to spread and invade surrounding tissue, the tumour is considered malignant. In the following sections, the development of cancer as a microevolutionary process will be discussed. This process occurs much more often and proceeds much faster than the evolution of species in an ecosystem, but it depends on the same principles of mutation and natural selection.

In the case of bladder cancer, as for many other cancers, the origin of the tumours is said to be multifactorial, with tobacco smoking as the principal cause in most countries (**see below**). The molecular development of the disease is highly complex and involves many genetic abnormalities. These abnormalities yield phenotypic changes that allow normal transitional cells to become cancerous and finally acquire the malignant phenotype. Some of the genetic changes can additionally be detected among a wide range of other malignancies, suggesting the existence of common 'key pathways' in cancer development, while others may be specific for bladder cancer. Besides providing an overview of the significance of bladder cancer, the following chapters also review the most common genetic alterations as well as the molecular mechanisms and pathways involved in the disease.

1.1 Epidemiology: significance of cancer in general, and of bladder cancer in particular

According to the WHO, cancer was estimated to account for about 7 million deaths (12% of all deaths) worldwide in 2000, only to be preceded by cardiovascular diseases (30%) and infectious and parasitic diseases (19%). Estimates for global cancer incidence and mortality are shown in **table 1**.

Urinary bladder cancer contributes significantly to the overall human cancer burden. Over 300'000 people are confronted with the disease and more than 130'000 patients die because of bladder cancer every year (**figure 2**). As far as modern societies are concerned, however, the incidence and mortality rates are somewhat higher than in developing countries. In the U.S.A. bladder cancer accounts for about 6% of all new cancers and is three times more common in men than in women (making it the 4th most common cancer in men). Furthermore, bladder cancer is twice as common in white individuals as in blacks. On average patients are 65 years old; only 1% is less than age 40.

Site	Incidence	Mortality
Trachea, bronchus and lung	1'211'804	1'089'258
Breast cancer	1'017'207	371'680
Stomach cancer	950'319	714'452
Colon and rectum cancers	944'677	510'021
Liver cancer	554'344	536'904
Prostate cancer	536'279	202'201
Cervix uteri cancer	474'387	232'153
Mouth and cropharynx cancers	462'979	250'900
Lymphomas and multiple	405'995	236'494
Oesophagus cancer	386'612	350'841
Bladder cancer	**326'532**	**131'681**
Leukaemia	255'932	209'328
Pancreas cancer	201'506	200'865
Ovary cancer	188'482	114'488
Corpus uteri cancer	185'951	44'359
Melanoma	131'469	37'654
Other sites	1'678'413	1'027'317
Total	**9'910'878**	**6'260'596**

Table 1: The incidence and mortality of cancer. Data are according to the *WHO 2000*. Total new cases of bladder cancer diagnosed were 326'532. Note that approximately 40% of the patients die as a result of the disease.

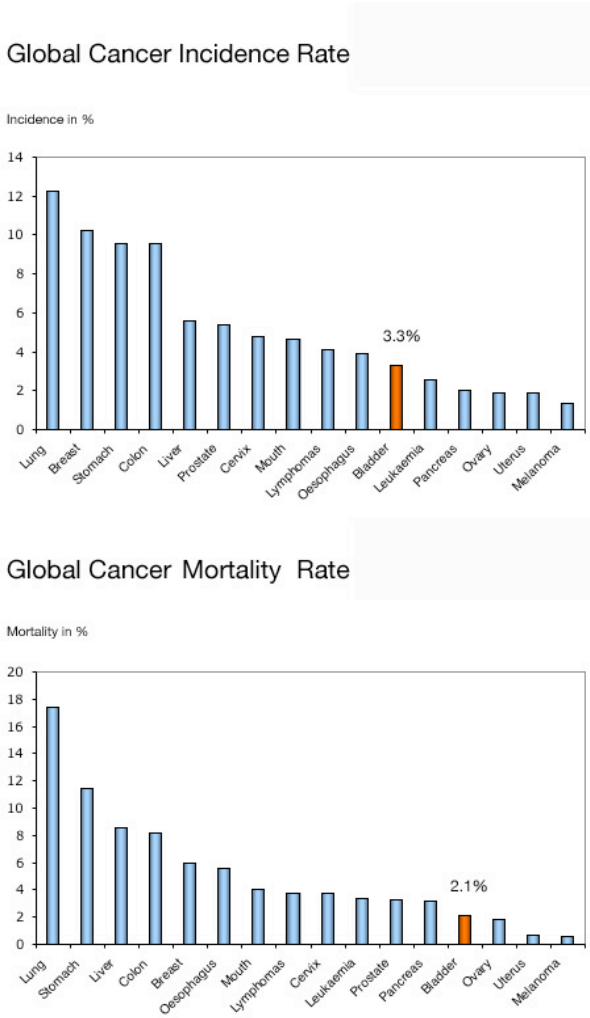

Figure 2: Global cancer incidence and mortality rates (according to estimates of the *WHO 2000*). Bladder cancer is accentuated (orange bars).

The reason why one person develops bladder cancer while others don't, is not yet fully understood. But several risk factors have been noted: cigarette smoking, diet (especially fried meat and fat), being old, being male, carcinogen exposure, family history, place of residence, some medications, skin colour, presence of bladder polyps, presence of chronic irritations of the bladder, or infection with a parasite (*Schistosoma haematobium*). Furthermore, workers with an increased risk of developing bladder cancer include painters, hairdressers, machinists, printers and truck drivers, suggesting that there might be a variety of carcinogens waiting to be detected. Progress in the early detection and treatment of bladder cancer has improved prognosis, with five-year survival rates of 60-80%.

Early mortality has declined in modern human populations throughout the past several 100 years due to improvements in hygiene and nutrition **(6)**. But, as more people survive into old age, more people encounter the diseases associated with old age. As a result, particularly the risk of cancer has dramatically increased with respect to other relatively frequent diseases (see **figure 3**). The ageing of the population in developing countries, points to an even further increasing burden of cancer worldwide for the future.

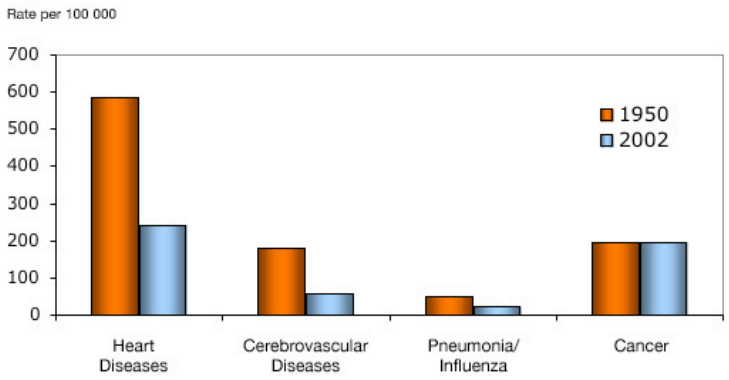

Figure 3: Change in US death rates by cause, 1950 and 2002. Compared to 1950, the cancer death rate in 2002 was about the same, while rates for other major chronic diseases decreased during the same period.

Source: 1950 Mortality Data – CDC/NCHS, NVSS. Mortality Revised.
2002 Mortality Data – US mortality public use data tape, 2002.

1.2 Histopathology of bladder cancer

Cancers can be classified according to their tissue and cell type of origin. Cancers arising from epithelial cells are termed carcinomas; those from connective tissue or muscle cells are termed sarcomas; and cancers that do not fit in either of these two categories are termed leukemias[1]. About 90% of human cancers are carcinomas, perhaps because most of the cell proliferation in the body occurs in epithelia, or because epithelial tissues are most frequently exposed to the various forms of physical and chemical damage that favour the development of cancer.

In bladder cancer, more than 90% of the tumours are pure transitional cell carcinomas (TCCs) or TCCs mixed with other histologies, primarily squamous cell carcinoma (SCCs), adenocarcinoma (ACs), or both. An additional 3% to 7% are pure SCCs **(8)**, which are approximately twice as likely to occur in women as in men. Besides, SCCs often develop in individuals who have *Schistosoma hematobium* infections of the bladder or who have histories of long-term indwelling urinary catheters, bladder stones, or recurrent bladder infections. Adenocarcinomas and undifferentiated cancers also occur, but much less frequently.

The degree of tumour extension is reflected by the 'staging system'. For example, small cancers in a single site are at an early stage, whereas cancers that have spread to many different parts of the body are at an advanced stage. Using the staging system has two main benefits. Firstly it gives an idea of how advanced a cancer is and secondly, it helps to determine what the treatment should be, since a patient with an early stage cancer needs different treatment than a person with a more advanced stage cancer.

Additionally to the tumour stage, a pathologist can assess the 'tumour grade', referring to the appearances of individual tumour cells under the microscope. Depending on how the cells look, a tumour may be given one of four grades. A low grade is when the cancer cells resemble normal cells, with only slightly abnormal changes (G1), while a high grade (G3) tumour contains cells that look very abnormal and show little or no resemblance to normal tissue. In between there is an intermediate grade (G2). The grade of a cancer is a guide to how aggressive the tumour is.

[1] Brain tumours do not entirely fit into one of these three distinct groups and have therefore to be classified separately (**7**).

Taken together, staging and grading help to predict how a cancer might behave, how it might respond to treatment, and what the chance of cure might be. An overview of all tumour stages that can be found in bladder cancer is given in **figure 4**.

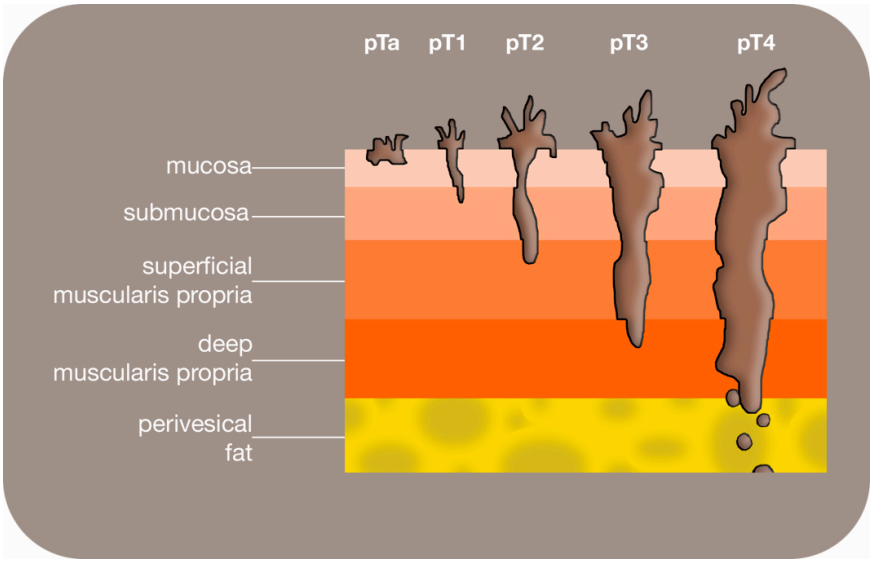

Figure 4: The different stages in bladder cancer. TNM classification: T= primary tumour; T0= no evidence of primary tumour; Ta= non-invasive papillary carcinoma; Tis= carcinoma in situ ("flat tumour"); T1= tumour invades subepithelial connective tissue, T2= tumour invades muscle; T3= tumour invades perivesical tissue; T4= tumour invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall; pT= pathologic classification (diagnosis after removal of tissue). According to *Bostwick & Lopez-Beltran 1999;* modified.

Approximately 75% of all bladder neoplasms are either non-invasive papillary tumours (pTa) or carcinomas with only minimal invasion (pT1). For the remaining 25%, the initial presentation involves muscle invasive disease that will usually relapse with metastases within a median of 2 years. The overall recurrence rates are high (30% to 83%), and even after instant treatment by resection and intravesical immunotherapy half of the pTa-pT1 TCCs recur within 4 years **(9)**. But the necessary repeated operative procedures to remove these TCCs are potentially morbid. Many recurrent papillary TCCs never progress to invasive TCCs **(10)**. However, a fraction of 20-30% may progress into muscle invading stages. Patients with tumours that develop to stage pT2-pT4 have a significantly worse prognosis.

Frequently, tumours staged as pT1 or less are grouped together by pathologists as "superficial bladder carcinomas" because they can be cured by transurethral resection in most instances. But, this classification is not very selective: in contrast to non-invasive tumours (pTa), the majority of the minimal invasive tumours (pT1) will progress into a more invasive phenotype (pT2-pT4). Since, the prognoses of patients and the choice of treatments depend on the aggressiveness and grade of the tumour, it would

be extremely important to find better criteria (genetic markers) for an accurate discrimination between different pT1 tumours **(10)**.

1.3 Cancer genes

Cancer results from an accumulation of mutations in genes called gatekeepers, caretakers, and landscapers **(11, 12)**. Gatekeepers such as oncogenes and some tumour suppressor genes directly regulate cellular growth and differentiation **(13)**. Oncogenes are activated by gain-of-function mutations that lead to increased or novel function; tumour suppressor genes, in contrast, are affected by loss-of-function mutations. Gatekeeper defects directly provoke abnormal cellular proliferation, differentiation, and apoptosis. Caretakers are a special group of tumour suppressor genes, which function in maintaining the genomic integrity of the cell, regulating DNA repair mechanisms, controlling chromosome segregation, and monitoring cell cycle checkpoints **(1)**. Caretaker defects lead to genetic instabilities that contribute to the accumulation of mutations in other genes, instead of directly affecting cell proliferation and survival **(14)**. Loss of caretakers is not essential for the development of cancer, but it accelerates the process. Finally, landscaper defects do not directly affect cellular growth, but generate an abnormal stromal environment that contributes to the neoplastic transformation of cells.

1.3.1 Molecular biology of cancer: How a normal cell becomes a tumour cell

Tumours derive from single somatic cells and their progeny. The cells in the emerging neoplastic clone accumulate a series of genetic or epigenetic alterations that lead to changes in gene activity or -function, and consequently to altered phenotypes which are subject to selection **(12)**. Evidence for this theory comes from analysis of the chromosomes in tumour cells. Chromosomal aberrations and rearrangements are only present in the cancerous cells of the tumour itself, but cannot be found in the healthy tissue. However, it is almost impossible for a single genetic change to cause a tumour. Estimated 4 to 7 distinct rate-limiting genetic events are essential for the development of common epithelial cancers **(11)** (see **figure 5**). Each mutation thereby creates a cellular clone that is increasingly well adapted for autonomous growth in the host organism. Eventually, a cell population might evolve that can overcome the control of normal proliferation and territory and become a cancer. Accumulation of all necessary steps or “pathway events”, normally requires a relatively long period of approximately

40-80 years **(15, 16)**. In inherited cancer syndromes one event is already present at birth throughout all cells of the body and as a result, people with inherited predisposition often suffer from an especially early onset of the disease.

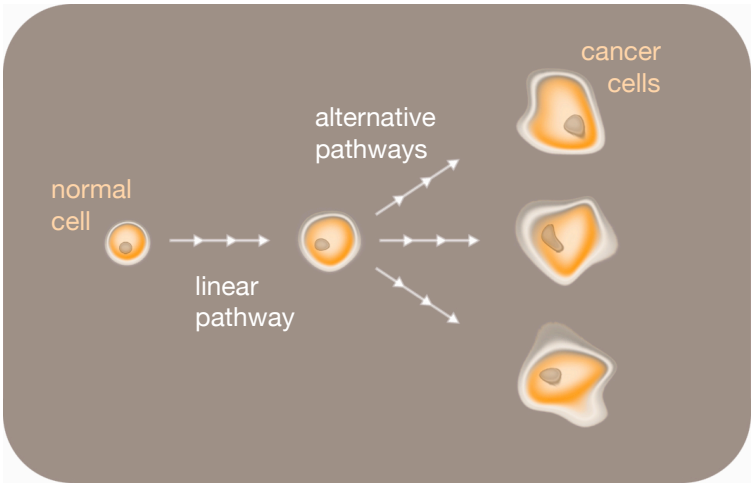

Figure 5: The "multistep model" as developed by *Hanahan & Weinberg 2000*. Specific associations of pathway events are seen within individual tumours, and these presumably reflect the evolution of the tumours along particular pathways.

Albeit tumour cells need to overcome similar constraints on the road to cancer, the importance of the constraints varies widely between different tissues, and can be evaded by alternative approaches. For example signalling pathways can be disrupted at different points (**figure 5**). On the other hand, tumours sometimes share particular mutations (e.g. of p53, Her-2/Neu, BRCA1 and BRCA2). This indicates in return, that some genes are especially important in tumourigenesis, independent from organs, tissues or patients.

1.3.2 Tumour initiation

The transformation from a normal cell to a malignant phenotype is a complex process that involves the interaction of many genes, proteins, and other molecules. Environmental chemicals are thought to play a predominant role in tumour initiation, perhaps giving rise to 70% of the mutations **(1)**. To add on, carcinogens either derived from occupational exposures, inflammatory conditions, or schistosomal infections are clearly among the most important environmental factors in bladder cancer initiation (see **above**).

From a biological point of view, tumour initiation starts with a genetic change in the DNA. Although shepherded with utmost care, DNA nevertheless is a molecule whose chemical bonds obey the same laws as other chemicals existing in an aqueous environment at 37°C in the middle of a cell and dependent upon making and breaking chemical bonds. Thus, DNA constantly suffers chemical damage, some as a consequence of chemical attack by reactive molecules leading to errors in the correct reading of the damaged DNA by DNA-poymerases, others are simply occurring as a result of errors in replication. The latter are also called spontaneous mutations. It has been estimated, that they are extremely rare. In cultured human cells the spontaneous mutation rate is only approximately 2×10^{-7} per gene per cell division **(16, 17)**. Therefore, it is insufficient to explain the incidence rates of cancer. Even if the extrapolated number represents an accurate measurement of *in vivo* mutations in a healthy body, extrapolation over a life-time would predict much lower prevalence of cancers than currently experienced.

A more realistic prediction arises from the scenario that genome stability will be continuously decreased by every further mutational event, leading to an accelerated pace of mutations overall **(18)**. This model particularly takes into consideration the well-recognized inherent instability of tumour genomes relative to their normal counterparts. It is important to note, however, that chemical damage itself is not yet a mutagenic event. DNA replication and subsequent cell division are additionally necessary to convert chemical damage into inheritable changes. Besides, chemical damage that does not enhance proliferation will not influence cancer development, since such cells have a high probability of being displaced by adjacent normal cells, sooner or later.

Many different mutations have been documented so far, but not all of them have the potential to induce transformations. A vast majority does not lead to truncated protein products, because approximately 97% of the DNA is non-coding. Others fail to affect protein expression as a result of the similar chemical properties of the interchanged amino-acids. Last but not least, a couple of mutations does not even give rise to amino acid substitutions due to the redundancy of codon recognition, proofreading capabilities of high-fidelity DNA polymerases and DNA-repair mechanisms. Thus, only very few mutations can overcome all these barriers and might finally provoke a genetic change.

Once mutagenic effects successfully destroy or encrypt the gene's instructions for making the protein, the affected cell will be subject to natural selection. If the genetic change enhances proliferation, a mutant clone will slowly but surely overgrow adjacent

cells (see **figure 1**). Cells harbouring heritable somatic mutations must therefore be regarded as transformed. Mutations with a particularly high probability of producing a transformed cell can be expected among tumour suppressor genes and oncogenes.

1.3.3 Tumour progression

Once an inherited mutation has increased the proliferation rate in a particular cell, tumour progression starts as an inevitable and logic process, following the basic rules of natural selection. As soon as one of the initially transformed cells in the developing clone will experience another advantageous mutation, perhaps further enhancing proliferation speed, facilitating cell cycle entry, improvement of nutrient supply, or else, its progeny will again overgrow the surrounding tissue as the process of clonal evolution goes into the next successive round (see **figure 1**) until the 'adult' primary tumour will finally gain the potential to distribute its metastatic deposits **(19)**.

In general, the rate of evolution in any population is affected by four parameters: (1) the mutation rate; (2) the number of individuals in a given population; (3) the rate of reproduction; and (4) the selective advantage provided by a genetic change. These factors are in the same way critical for the evolution of cancer cells in a multicellular organism. Suggesting that a minimum of 4-7 individual mutations are needed for the development of a normal cell into a malignant tumour, it is not surprising that the risk for cancer dramatically increases with age **(6)** (see **figure 6**).

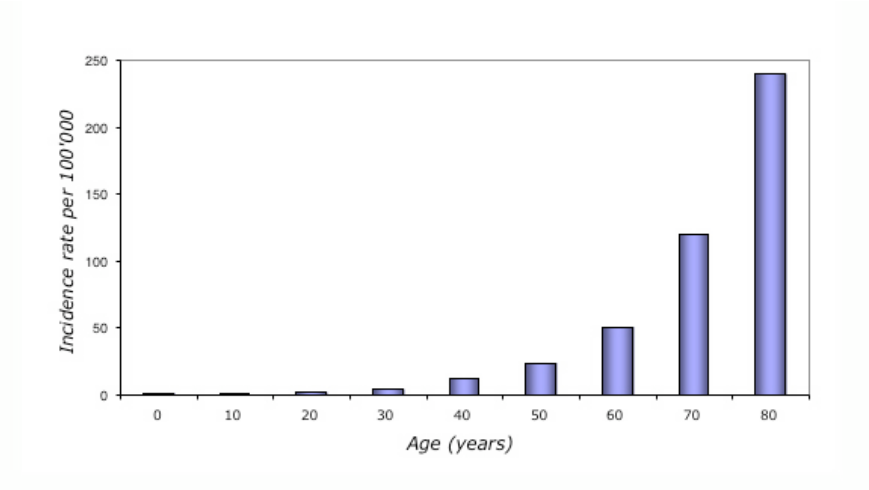

Figure 6: The cancer incidence rate dramatically increases with age. The current data support the theory that cancer is a multifactorial process. Development of the disease cannot be triggered by one single mutation but instead needs at least 4-7 distinct somatic mutations. Mutations arise equally often throughout life and accumulate until, at a certain age, the disease breaks out. If a single mutation was sufficient to cause cancer, incidence rates would be expected constant throughout all age classes. Source: *IARC, 1987.*

Furthermore, it is obvious that, perhaps as a result of the mutational events in combination with disturbed epigenetic regulation, altered stromal milieu, and telomeric instability, the accumulation of subsequent genetic changes during tumour progression will be further facilitated.

Like cells of distinct tissue types, bladder cancer cells need to acquire some particularly important "major" pathway events (**figure 7**) in order to be able to grow more rapidly, invade, and metastasize. These essential properties include uncontrolled growth and cellular mobility, mediated at least in part via growth factors and their receptors, expression or loss of expression of specific cell adhesion molecules, and overproduction of angiogenic factors.

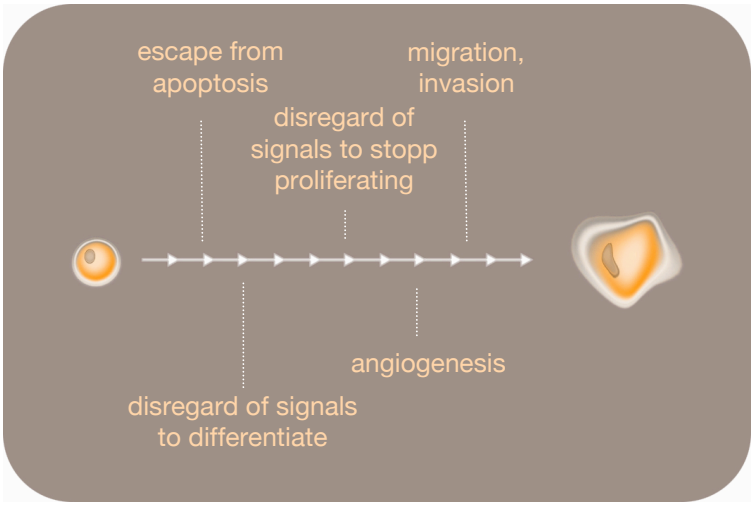

Figure 7: The conclusion that cancers result from a series of 4-7 independent somatic mutations is supported by studies of people who inherit a strong susceptibility to the disease. If single mutations were able to cause cancer, the chance of developing the disease should be independent of age. Above the "hallmark features of cancer" are depicted (modified according to *Kinzler & Vogelstein, Nature 1997*).

1.3.4 Inheritable factors affecting the development of cancer

As mentioned earlier on, a subset of the genetic changes leading to cancer are inheritable. For example in breast cancer, the risk of close relatives of a patient, averaged across all ages, is about 2-fold. Most of this familial risk is probably genetic in origin. The risk is about the same for the mother, sisters or daughters of a breast cancer patient, suggesting dominant rather than recessive effects. Large population-based studies indicate that only between 15-20% of overall familial risk is attributable

to the strong predisposition genes BRCA1 and BRCA2. The possibilities for the remaining 80% are some combination of a small number of moderately strong genes, and a larger number (possibly a hundred or more) of weaker tumour suppressor genes **(14, 20, 21)**.

For bladder cancer, however, the influence of inheritable factors seems to be quite low **(19)**. Only occasionally inheritable tendencies are detected in familial clusters (accounting for less than 1% of all cases). For example people with a mutation of the retinoblastoma gene, which causes cancer of the eye already during childhood, have an increased rate of bladder cancer. But apart from the retinoblastoma gene, there is currently no further evidence for inheritable genetic changes in bladder cancers **(22)**. It seems that common genetic polymorphisms (such as being white) have only modest effects on risk and mostly interact with known risk factors such as smoking (check above). Thus, as a general rule, the overwhelming majority of bladder cancer cases is sporadic and does not show familial patterns.

1.3.5 Epigenetic factors affecting the development of cancer

Work over the past years has shown that gene expression is not determined solely by the DNA code itself but additionally by the assortment of proteins and, sometimes, RNAs that tell the genes when and where to turn on or off. Such epigenetic phenomena enable cells to respond to environmental signals conveyed by hormones, growth factors, and other regulatory molecules without having to alter the DNA itself. It is obvious that e.g. monozygous twins are not fully identical, although they share a common genotype. Even in twins, some variable types of phenotypic differences are always present, including dissimilar susceptibilities to cancer and several other diseases. Molecular mechanisms that drop into the category of epigenetic effects are: genomic imprinting, gene silencing and DNA methylation. Epigenetic differences increase with age **(23)**. Observed twins were epigenetically indistinguishable during the early years of life, whereas older twin pairs exhibited remarkable differences in their overall content and genomic distribution of epigenetic factors, often visibly affecting their gene-expression portrait. This demonstrates how epigenetic mechanisms can form different phenotypes originating from the same genotype. The same holds true for cancer cells. They started out as identical twins of their neighbours that began to deviate. Wheeler and colleagues **(24)** recently reviewed that hypermethylation of gene promoters can efficiently inactivate tumour suppressor genes.

1.3.6 Tissue specificity and variability of expression

Local factors can strongly affect the development of cancer: If only the sequential accumulation of somatic mutations over a lifetime would finally drive a subset of cells in the organism over a critical threshold, leading to emergence of cancers late in life, the risk of developing a cancer should be equally high in all tissues. But cancer statistics clearly show a different picture (**figure 2**). All inherited predispositions to cancer exhibit a considerable degree of tissue specificity, even in the case of predisposition by defective DNA-repair. The exact mechanisms, however, still remain unknown.

Evidence for a crucial role of local gene expression comes from the sheer endless seeming list of substances with precise tissue specific effects: e.g. artificially increased oestrogene-levels, either induced through taking oral contraceptives or through hormone-replacement-therapy, enhance the risk for breast cancer, while the risk for reproductive carcinomas is significantly lowered **(14, 25)**. Apart from the tumours' local environment, also wounding and chronic inflammation can influence cancer development (chapter **1.1 Epidemiology**). Their effects may be mediated either through increased mitogenesis, which may be associated with increased mutation, or through paracrine effects. Again, it is also possible, that there will be genetically determined variation in the wounding and inflammatory responses between tissues and/or individuals.

1.4 Genetic alterations in bladder cancer

Generally, the development of bladder cancer is not much different from development of other malignancies, in a similar way characterized by tumour initiation and -progression. However, as a typical feature tumours of the urinary bladder are extremely heterogeneous with respect to their genetic alterations, histopathologic properties, morphologic growth patterns and clinical behaviour. Therefore, it must be expected that many different biological pathways could be involved in bladder cancer. An overview of frequent genetic changes encountered in bladder cancer is given in **table 2**.

genomic region	frequency (%) loss	gain	potential target gene(s)
9q	83		?
9p	55-78		?
8p21	54-65		DBC2, LZTS1
3p	56		?
13q14	20-56		Rb
10q	52-54		ERCC6, PTEN, and DMBT1
8q	50		?
17p13	45		p53
5p	43		?
5q	41		MCC; APC; IRF-1
11p15	40		CDKN1C/p57(KIP2)
1p36		84	p73, TNFRSF12, E2F2, FGR
11q12-q23		36-78	CCND1, EMS1, PRAD1
12q13		18-77	MDM2, GLI, SAS, CDK4, TIP120A
17q21		73	ERBB2
6p22		60	E2F3, CDKAL1, SOX4, ID4
8q21.3-q22		54	?
8q24		45	MYC
7p		45	EGFR
20q		40	AIB1, BTAK, NABC1, ZNF217, BCAS4

Table 2: Most frequent genetic alterations in bladder cancer (by CGH; FISH). Source: *Reznikoff et al., 2000; Terracciano et al., 1999 Hurst et al., 2004, Zaharieva et al., 2003; Simon et al., 2003, Simon et al., 2002; Simon et al., 2001; Oeggerli et al., 2004; Genome Database 2005.*

Data from comparative genomic hybridization studies (CGH) have lead to the identification of many chromosome region losses and gains in human bladder cancers (**table 2**). The non-random losses and gains of chromosomal DNA during tumourigenesis have been generally interpreted to signify the contribution of inactivation and activation of 'cancer genes' to tumourigenesis. Losses are most likely to be involved in the inactivation of tumour suppressor genes, whereas gains may be a means to activate oncogenes.

Since the majority of non-random losses and gains have been known for years, many relevant cancer genes are already identified. The list of prominent examples is long: loss of p53 (at 17q13), Rb (at 13q14), and PTEN (at 10q22), or alternatively amplification of Her2/Neu (at 17q21), CCND1 (at 11q13), and EGFR (at 7p12). However, critical gene(s) for some chromosome regions remain(s) unknown, including: 4q, 9p, 1p36, 20q and 6p22. According to current data, 6p22 represents the 2nd most frequently amplified chromosome region in human bladder cancer (as depicted in **Figure 8**).

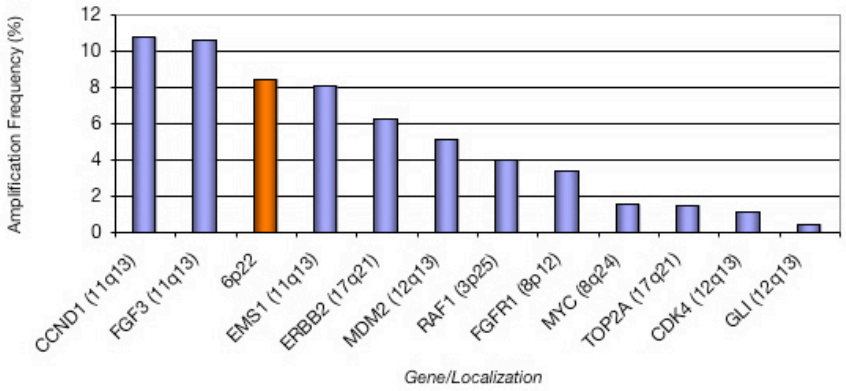

Figure 8: The genomic region 6p22 is frequently amplified in human bladder cancer. Amplification hot-spots determined by fluorescence *in situ* hybridisation are displayed above. Source: *Simon et al., 2001, 2002 and 2003, Oeggerli et al., 2004.*

1.4.1 DNA amplification

Gene amplifications occur in variety of organisms ranging from bacteria to mammals. It can be a naturally regulated mechanism (ribosomal DNA amplification in amphibian oocytes) but it can be also an aberrant process that occurs uncontrolled in genetically unstable cells. In the recent years, DNA sequence amplification has been intensively investigated in connection with drug resistance and tumourigenesis in mammalian cells **(26)**. The first observation of DNA amplification was the identification of distinct chromosomal structures termed homogeneously staining regions (HSRs) and double minute chromosomes (DMs) in the cytogenetic analyses of a drug resistant cell line. **(27)**. Further accumulation of evidence suggested that, while HSRs are often found in human tumour cell lines in culture, the prevalent manifestation of amplification in human tumour cells is in the form of DMs **(26)**. The hallmarks of most aberrant amplification phenomena is that the amplified DNA sequences (amplicons) are arranged end to end in tandem arrays either in chromosomes (HSRs) or in exrachromosomal elements (DMs). It was suggested that amplicons could have complex patterns due to co-amplification of non-contiguous parts. Such a discontinuity indicates that numerous rearrangements take place during evolution of the amplicon **(28)**. Additionally, an amplicon from a given locus can be extremely variable in size and position, both among different cell lines and of the same species and within the same cell line. Another interesting fact is that the copy number of

amplified sequences is not uniform across the entire amplicon. This suggests that only a part of the original amplified unit is amplified in subsequent steps. The corollary of amplicon heterogeneity is that amplicon endpoints apparently do not represent hot spots for the recombination events that join the amplicons together. In tumours the level of amplification may vary from few to more than 1000 copies but more than 50-fold amplification in primary tumours is rare. The size of the amplified DNA segment varies considerably from approximately 50 kb to 50 Mb suggesting the existence of micro amplicons that can not be detected by comparative genomic hybridization (CGH) due to the limited resolution and dynamic range of this technique **(26)**.

The mechanism of gene amplification in human cancer cells is still unknown, but several different models have been proposed. The basic starting point by all proposed models is the fact that a cell starts out with two copy loci and ends up with more than two after the first step, so appearance of this extra DNA is a subject that needs to be explained. The outlined models invoke either over-replication, disjunction or some combination of these processes.

Bridge-breakage-fusion model. The initial event is chromosomal breakage followed by fusion of the broken pieces and formation of dicentric sister chromatides. This model explains the presence of amplicons with different sizes and molecular configurations. Recently breakage events at chromosomal fragile sites have been seen to be involved in DNA amplification **(29)**.

The episome-excision model. Extrachromosomal amplification may be generated by the formation of small circular acentric molecules that can multimerize to form DMs **(29)**.

Unequal exchange model. Multiple gene copies are generated by recombination events between homologous and non-homologous DNA sequences on two misaligned chromosomes or chromatides **(30)**.

1.4.2 Oncogenes activated – tumour suppressor genes inactivated

Oncogenes are defined as a gene, whose activation allows uncontrolled growth and causes normal cells to become cancerous. As a logical consequence, oncogenes are found amplified and overexpressed in many types of cancers, including tumours of the urinary bladder. Oncogenes that are frequently found amplified in bladder cancer have been suggested to be involved in pathogenesis of the disease. Gene amplification is the most common mechanism for their activation rather than translocation or mutation.

Many of these suspected genes encode for growth factor receptors or factors involved in gene transcription or cell cycle regulation.

One potential prognostic marker for bladder tumour progression is the epidermal growth factor receptor, encoded by the gene ErbB1 (synonym EGFR) located at 7p13. EGFR is activated by binding of ligands, epidermal growth factor (EGF), **(31)** and transforming growth factor alpha (TGF-alpha) to its external domain **(32)**. This activation results in autophosphorylation of Egfr protein by the cytoplasmatic portion of Egfr and subsequently leads to cellular proliferation, transformation and cell division. Immunohistochemistry studies have shown a significant correlation between EGFR expression, grade and stage, as well as association with tumour cell proliferation and progression. EGFR gene amplifications were found in 10-20% of Egfr overexpressing bladder carcinomas. **(33)**

The HER-2/Neu oncogene, located at the chromosome 17q21 encodes a transmembrane glycoprotein that is closely related to the epidermal growth factor receptor (EGFR). Overexpression of the HER-2 protein is a frequent event and has prognostic value in a variety of human tumours **(34)** HER-2 has been found amplified and overexpressed in high grade and stage TCCs **(35)**.

Furthermore, several studies have reported that HER-2 expression in patients with bladder cancer is associated with increased incidence of metastasis **(36)** and decreased overall survival **(37)**. Although these studies suggest a prognostic value of HER-2 expression, others have reported conflicting results, concluding that evaluation of Her-2 provides no additional prognostic value over previously established predictors (stage and grade) for transitional cell carcinoma of the bladder **(38)**.

The MDM2 proto-oncogene (murine double minutes) product acts as a major regulator of the tumour suppressor p53 by promoting its degradation **(39)**. MDM2 was mapped to the 12q14.3-q15 chromosomal region by FISH distal to CDK4 gene. Amplification of MDM2 gene is sporadically reported in bladder cancer **(40)**. Another gene candidate, CDK4 resides at the same genomic region and is recurrently amplified in bladder cancer **(41)**. Its role in the cell cycle at the G1-S checkpoint is well documented. Regulation of CDK4 is controlled by CDKN2A ($p16^{INK4A}$) another key regulator in bladder cancer biology. The 12q13-15 amplicon contains potential target genes involved in cell cycle regulation and comprehensive study of this amplicon detected co-amplification of MDM2 and CDK4 in (10.6%) of analyzed tumour samples. Immunohistochemistry revealed conflicting results linking MDM2 expression to high, or

to low grade and stage tumours **(42)**.

11q13 represents another region that is extremely often amplified in bladder cancer. It harbours the gene CCND1 (Cyclin D1) which is involved in the regulation of G1/S phase transition of the cell cycle **(43)**. Several studies have indicated that CCND1 overexpression occurs in urinary bladder cancer and that its expression is associated with low grade, low stage, and papillary tumour growth **(44)**. However, the gene revealed no prognostic significance for increased CCND1 overexpression in superficial urinary bladder cancer **(45)**.

The MYC gene, mapped to chromosome 8q24, encodes a nuclear phosphoprotein involved in transcription regulation. Its normal function has been linked to growth regulation, cell differentiation, and apoptosis **(46)**. The few studies characterizing MYC protein expression in bladder cancer have yielded controversial results, showing protein overexpression associated with low stage tumours **(47)**, as well as with high grade tumours **(48)**. A study using FISH and immunohistochemistry suggested that c-MYC protein overexpression is associated with low grade and early stage of bladder cancer and that MYC gene copy number gain is more frequent in advanced tumours. A common mechanism for activation of MYC family genes is chromosomal translocation. A recent report describes similar findings, regarding MYC mRNA **(49).** However, a significant correlation was found between the methylation pattern and mRNA overexpression of the MYC oncogene in bladder tumours **(50)**. Therefore it appears that mechanisms other than amplification may as well cause MYC protein overexpression in bladder cancer.

The H-RAS gene is an oncogene thought to be involved in the development and progression of bladder cancer **(51)**. Mutation analyses of the RAS gene family have demonstrated alterations in codon 12, 13 and 61 of the H-RAS gene **(52)**. H-RAS belongs to a family of plasma membrane associated signal transduction molecules. Activated H-RAS interacts with a large series of downstream signal molecules with multiple functions including the stimulation of proliferation.

Alternatively to increased activation or overexpression of growth factor receptors, tumour growth could also benefit from inappropriate ligand concentrations. Thus, ligands also possess oncogenic potential (e.g. TGF-alpha, PDGF, EGF). However, the ultimate target of the oncogenes is to achieve activation of transcription factors such as c-myc or E2F3. The more direct activation circumvents the complexity and feedback controls which exist in upstream signal transduction pathways. Evidence for

this theory comes from c-myc, a transcription factor whose expression is tightly regulated in normal cells and is only expressed in S-phase of the cell cycle. The chromosomal region harbouring c-myc (8q24) is frequently amplified in a large number of bladder cancers. Additionally, the gene is overexpressed throughout the cell cycle in a large number of human tumour types, driving the cells continuously towards proliferation. If the tumour suppressor genes that normally restrict this action by initiating apoptosis are mutated themselves, inappropriate proliferation occurs.

Genes whose inactivation plays a role in oncogenesis have been termed tumour suppressor genes. Whereas in oncogenes, mutation of a single allele is sufficient to induce the oncogenic function because of the dominant nature of the activation step, both alleles must be inactivated in tumour suppressor genes. Loss of only one allele of a suppressor gene is generally silent and allows germ-line inheritance of the damaged allele. Familial inheritance of mutated tumour suppressor genes can lead to cancer-prone individuals. The study of such inheritance patterns has allowed significant breakthroughs in the identification of responsible genes.
In particular, loss of 3 tumour suppressor genes by deletion, mutation, and/or methylation-inactivation seems to be of unquestionable importance in developing bladder cancer cells: Rb at 13q14 is altered in 30% TCCs, cyclin dependent kinase inhibitor gene (CDKN2A/p16) at 9p21 is altered in 20-60% TCCs and in 70% SCCs and p53 at 17q13 is altered in 50-60% TCCs and SCCs.

Last but not least, inactivation of DNA repair genes leads to increased genetic instability and mutation rates. Defects in DNA repair systems such as, nucleotide-excision repairs and mismatch repair have been well documented in cancer **(53)**.

1.5 How can evolutionary theory contribute to understanding cancer?

Just as Darwinian evolution depends on random mutations leading to genetically different individuals, it now seems clear that random mutations of the genes, which regulate proliferation or control apoptosis are responsible for cancer. To take the analogy further, just as natural selection allows the survival of the fittest individual, in the case of carcinogenesis it will instead select the fastest growing cells, if they manage to overcome a few limiting processes. The transformation that allows a normal cell to become a malignant one is now widely accepted to be a stepwise process and does normally require between 40 to 80 years **(16)**. Cancer is typically recognized as a disease of the elderly. According to the concepts of Darwinian evolutionary theory,

natural selection must be expected to enhance adaptedness to prolonged life. Thus, at first sight, it might seem puzzling why better mechanisms to avoid deleterious mutations did not evolve. However, as one can easily imagine, it is almost impossible to construct and operate a highly complex replicational process that functions 100% error-free. Additionally, such a 'perfect' system would also fail due to basic theoretical principles of Darwinian evolution (no more variation among individuals; stop of evolutionary processes; extinction of species).

To completely understand incidence and mortality rates and the disease 'cancer' as a whole, it is important to consider the mechanisms causing the decline with age –often described as 'senescence'. This chapter, therefore, is devoted to describe the theories that have been proposed to explain cancer and the evolution of senescence.

1.5.1 Brief introduction to the theory of senescence

So far, our focus has been on the developing cancer cell, on the genetic events and the deficiencies in DNA repair and genomic stability, which drive the process. But this focus provides only part of the picture. It is likely that genetic variation at other sites, within and outside the cancer cell, may substantially affect cancer development. Darwinian investigations of the evolution of senescence start from the observation that, in nature, most animals die before they reach old age. As a consequence, genetic mutations that have their effects on adaptation late in life are selected against only weakly, in comparison with mutations affecting fitness early in life.
Imagine a mutation that increases survival or fecundity early in life, at the expense of reducing adaptedness late in life. Such a mutation would be favoured by natural selection. There are many reasons why such trade-offs between success early and late in life are to be expected. Experimental evidence demonstrates that trade-offs do not only exist in theory, but also in reality: e.g. in *Drosophila* almost any environmental treatment (including 5000 rad of X-rays) that reduces the rate at which a female lays eggs prolongs her life. On the other hand side, artificial selection for increased longevity reduces early fecundity **(54)**. There are currently three reasonable explanations for the seemingly high incidence and mortality of cancer, without being necessarily exclusive:

1.5.2 Influence of stochastic processes

Many different stochastic processes, eventually leading to senescence, are suspected to play a role in formation and progression of cancers: e.g. changes in the nuclear genome, erroneous transcription and translation and oxidative damage. The trade-off model predicts, that even if all these processes could be eliminated (by more accurate replication and translation, or more effective removal of oxidizing agents) a trade-off would exist, and energy employed to protection would not be available for reproduction, if maintenance mechanisms are costly. And it is obvious that such costs must exist.

1.5.3 Influence of mutant-genes

It is well documented that mutant genes exist in populations **(55)**. Each is likely to be at low frequency, maintained by a balance between mutation generating new mutant alleles, and selection from time to time eliminating them. The selection against a gene whose effects on fitness are late in life will be relatively weak, and its frequency in the population correspondingly high, even if it has no counterbalancing beneficial effects early in life. Thus we expect late-acting deleterious alleles to accumulate, and eventually to cause cancer. After all, genome instability is playing a fundamental role also for the long-term survival of existent species. The evolution of the human race would have been much slower if it had only involved genetically stable individuals. From the individual's point of view, however, it would be advantageous to have maximal genetic stability.

1.5.4 Influence of the environment

It is often stated that humans are not fully adapted in many ways to current lifestyles and to such a long life expectancy as normally experienced in modern human populations **(56)**. Lack of adaption is generally made responsible e.g. for woman's reproductive cancers. Typical members of our modern society experience earlier menarche, late first birth, less nursing, lower parity, and later menopause **(57)**. The net effect is to increase the exposure of reproductive tissues to oestrogenic hormones, which in turn increases cell proliferation (e.g. breast duct cell turnover rates are up to 20 times higher between menarche and first birth than after the first full-term

pregnancy). Cells that are dividing frequently are more likely to develop clinical malignancy. Information about women's cancer rates in foraging societies is unknown, but it may well be that women in modern societies have a risk that is from 10- to 100-fold greater. Nevertheless we know, that menarche delay, early pseudo-pregnancy, and oestrogen-lowering oral contraception are reducing the risk of reproductive cancers, perhaps by recreating an ancestral hormonal milieu **(25)**. It has been suggested that modern lifestyles may also increase the risk for bladder cancer **(58)**. Recently found risk factors for bladder cancer include compounds present in hair lotions (arylamine), painkillers (phenacetine, chlornaphazine and cyclophosphamide, formerly added to Aspirin® and Paracetamol®) as well as drugs momentarily utilized for chemotherapy and arsenic drinking water. Last but not least, smoking remains to be the highest risk factor. Cigarette consumption probably accounts for almost half of all bladder cancer induced deaths in the USA (48% for men; 28% for woman; according to US National Cancer Institute). Some of the cancer-causing chemicals in tobacco smoke are absorbed from the lungs and get into the blood. From the blood, they are filtered by the kidneys and concentrated in the urine. Since the urine is stored in the bladder, carcinogens can cause irreparable damage to the urothelial cells that line the inside of the bladder. This damage increases the chance of developing cancer. Risk is reduced with cessation of smoking, but a relatively small decrease in incidence is seen for the first 5 to 7 years after cessation. Even after 10 years, the risk of an individual developing bladder cancer is still almost twice that of an individual who has never smoked. Among the chemicals implicated in smoking-induced bladder cancer are aminobiphenyl. It is, like many other chemicals, only becoming an effective carcinogen after being metabolized by detoxifying enzymes. As a conclusion, there are several harmful agents increasing the risk of bladder cancer. Sadly, the majority are explicitly accumulated by modern lifestyles.

1.6 Finding cancer-critical genes

Finding critical genes for mutant phenotypes is already quite difficult, but for cancer the task is particularly complex because a typical tumour possesses a whole set of mutations. Moreover, due to the genomic instability of cancer cells, it is a challenge to detect those genetic, chromosomal, and transcriptional changes that are fundamental to the malignant process versus those that represent secondary or epigenetic aberrations **(1)**. In general, detecting the latter might be valuable for prognostic purposes, while identification of the former are useful to develop preventive strategies. However, awareness of both is essential to develop effective therapeutic approaches.

Many cancer relevant genes are already identified -but at the same time it is clear that many more will follow. A few specialised methods are preferentially utilized for their detection. In the case of dominantly acting oncogenes, successful identification can be achieved by a cell transformation assay. This method starts by scanning the genome of the cancer cell for fragments of DNA that will, when introduced into cells of a suitable tester cell line, drive them toward cancerous behaviour. Therefore, DNA must be extracted from tumour cells, broken into fragments, and introduced into the host cell line in culture. If any of these segments contains an oncogene, small colonies of abnormally proliferating transformed cells may begin to appear. The formerly introduced fragment must then be isolated and sequenced to see if a gene is mutated. The Ras gene, which is probably mutated in one out of four human tumours, was identified using this strategy.

As it was the case for the retinoblastoma tumour suppressor, a method to find cancer-relevant genes is to analyse the inheritance pattern of a cancerous disease **(59)**. Retinoblastoma is a childhood cancer that accounts for approximately 1% of cancer deaths in children. 40% of all cases are inherited. Affected individuals often develop the disease shortly after birth. Additionally, there is a high probability of the second eye becoming involved within 4 years and/or the development of other malignancies. In contrast, most of the sporadic cases have only a low incidence of involvement of the second eye and there is no enhanced risk for other malignancies. This pattern convinced Knudson to hypothesize a two-hit theory of carcinogenesis. Subsequent family studies and analysis of patients with inborn chromosome abnormalities, lead to the detection of the 13q14 genomic region **(60).** Using the known location of the chromosomal deletion associated with the disease, it was possible to clone and sequence the gene whose loss appears to be critical for the development of the cancer –the retinoblastoma **(Rb**). Following its detection, the gene turned out to be also missing in many other common types of cancer, indicating that loss of Rb is a major step in the tumourigenesis (see **figure 9**).

Another method -loss of heterozygosity analysis (LOH) -has largely helped to identify the localization of many tumour suppressors. Known and candidate genes identified by LOH include CDKN2A, DBC1 and TSC1 on chromosome 9, PTEN on 10q22, Rb on 13q14 and p53 on 17q13 **(61)**.

Following the years of Rb-detection, however, Knudson's theory received additional support from comparative genomic hybridization (CGH) studies, which is a good

technique to detect large chromosomal abnormalities **(62).** Gene amplification is a common mechanism for upregulation of oncogenes. Yet, more than 30 different loci have been identified that harbour DNA amplifications **(63-65)**. Furthermore, CGH enabled the identification of many regions that contain known or candidate oncogenes (see **table 2**), including: cyclin D1 (CCND1) at 11q13 **(66)**, ERBB2 at 17 q21 **(67)** and MDM2 and CDK4 at 12q14-q15 **(41)**.

Introduced in the late 1980's, Fluorescence *in situ* hybridisation (FISH) is now often used to detect or confirm gene or chromosome abnormalities, alternatively. Utilizing fluorescence labelled DNA probes has the advantage that even relatively small amplifications beyond the resolution of routine cytogenetics are visible **(68)**.

Last but not least, telomerase activity is present in most human malignant tumours, whereas it is generally not detectable, in normal cells. Therefore, it represents an alternative tool for tumour detection that is non-invasive. Telomerase activity can usually be measured by using a telomeric repeat amplification protocol (TRAP) assay **(69)**.

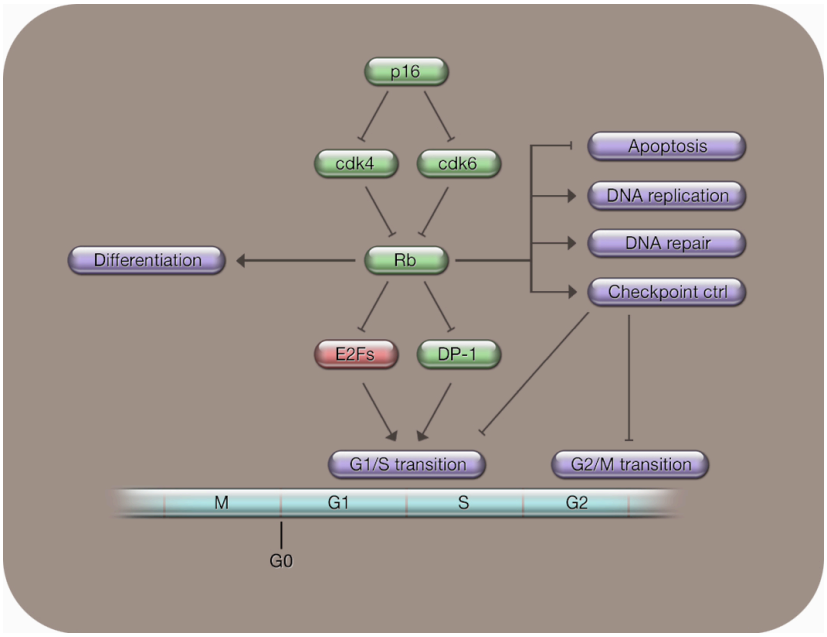

Figure 9: Deregulation of Rb is a common event in tumourigenesis. Rb affects many important cellular mechanisms. Alterations in cell-cycle regulatory genes that encode proteins that participate in the regulation of Rb function are also commonly observed in a broad spectrum of tumour type. *(acc. to Classon & Harlow, Nature 2002; modified) .*

Despite nowadays a broad range of different methods is available to find cancer genes, corresponding target genes remain unknown for the majority of amplicons,

such as for 1q21-31, 2q13, 3p22-24, 6p22, 8p11, 8q21, 9p21, 10p13-14, 13q13, 13q31-33, 18p11, 20q, 21p11, 22q11-13, Xp11-13 and Xq21-22.2 **(64, 65, 70, 71).**

1.6.1 Chromosomal region 6p22

Chromosome 6 constitutes about 6% of the human genome. The finished sequence comprises 166,880,988 base pairs, representing the largest chromosome. The entire sequence has been subject to high-quality manual annotation, resulting in the identification of 1,557 genes and 633 pseudogenes. At least 96% of the protein-coding genes have been identified, as assessed by multi-species comparative sequence analysis, providing evidence for the presence of further, otherwise unsupported exons/genes. According to the chromosome 6-research database (**www.sanger.ac.uk**), some of the uncharacterized genes might be implicated in cancer, schizophrenia, autoimmunity and many other diseases. In addition, chromosome 6 presumably harbours the largest transfer RNA gene cluster in the genome. Furthermore, the essential immune loci of the major histocompatibility complex (MHC), HLA-B, was found to be the most polymorphic gene on chromosome 6 and in the human genome.

Amplification of the chromosomal region 6p22 occurs in roughly 10-20% of bladder cancers, making 6p22 one of the most common sites of high-level amplification in this tumour type **(70, 72-76)**. Based on our previous comparative genomic hybridization (**CGH**) data of more than 300 bladder carcinomas, amplification of 6p22 was present in 10 of 172 advanced-stage tumours of our patients **(70, 77)**. Additionally, presence of 6p22-amplification correlates with the tumour grade **(70, 78)** and is therefore respected as clinically relevant.

1.6.2 Potential target genes of 6p22.3

It has been predicted that 6p22 must harbour one or more oncogene(s) that drive the amplification **(72, 76, 77)**. In order to define the borders of the amplification unit, the 6p22 region was FISH-mapped in our lab prior to this study **(73)**. Results narrowed down the minimal commonly amplified region to a 1.7 Mb interval (see **Figure 10**). The recently completed sequencing of the entire human genome now additionally allows direct information on genes that reside in the 6p22.3-core of the amplicon. According to this, the region of interest includes presumably 13 genes: Q9H1N9, PRL, SOX-4,

NM_017774 (=CDKAL1), E2F3, OACT1, ID-4, TFAP2, HMGIY, CCND3, IRF4, HOX12, and PIM1.

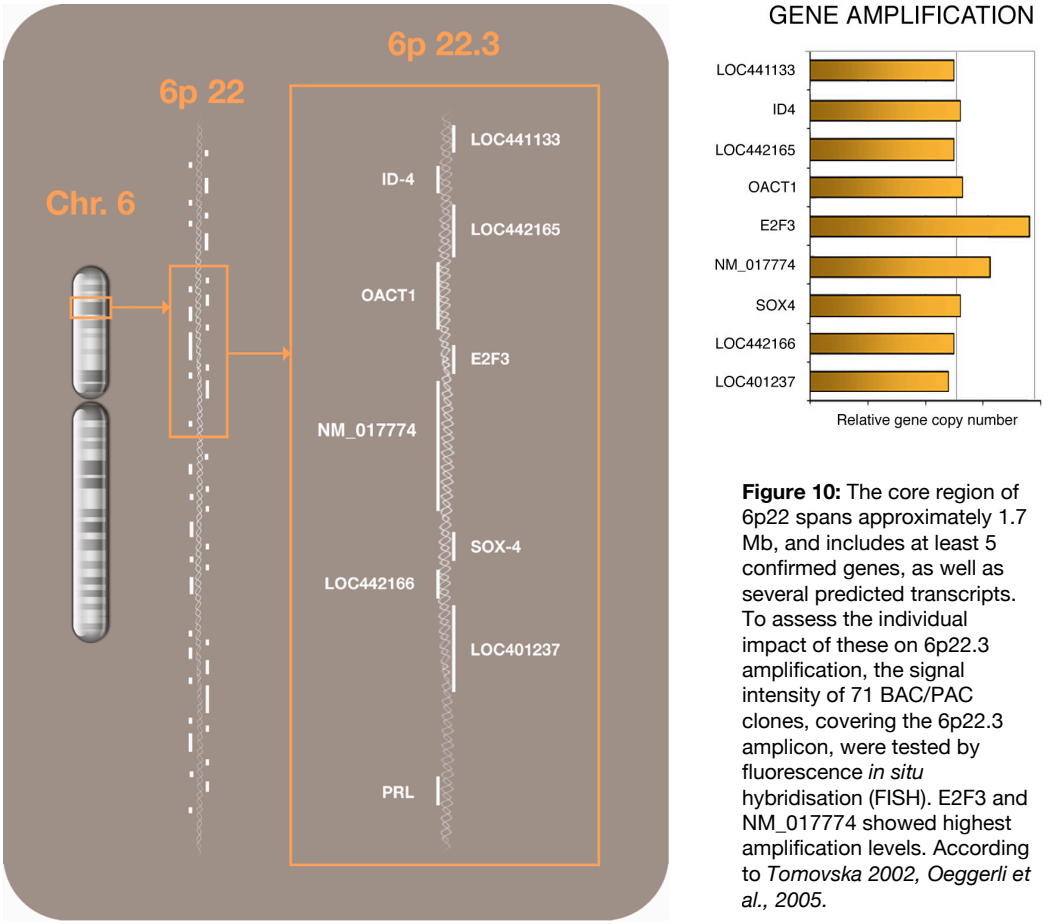

Figure 10: The core region of 6p22 spans approximately 1.7 Mb, and includes at least 5 confirmed genes, as well as several predicted transcripts. To assess the individual impact of these on 6p22.3 amplification, the signal intensity of 71 BAC/PAC clones, covering the 6p22.3 amplicon, were tested by fluorescence *in situ* hybridisation (FISH). E2F3 and NM_017774 showed highest amplification levels. According to *Tomovska 2002, Oeggerli et al., 2005.*

Prior to this work, a couple of genes were excluded as presumptive candidate oncogenes: SOX-4 and Q9H1N9 showed relatively weak expression, unrelated to 6p22.3 amplification **(74)**. And expression of PRL was to be found even completely absent in bladder tissues **(76)**.

In contrast, expression patterns of some other candidate target genes, suggests an involvement in 6p22.3-amplification. E2F3 was detected to be strongly amplified and overexpressed in a high percentage of human bladder cancers **(74, 76),** as well as in some prostate cancers **(79)**. However, in one report overexpression data favours NM_017774 (=CDKAL1) over E2F3 as responsible target gene **(74)**. As a consequence, the presumptive 6p22.3 oncogene remains to be conclusively identified. It is not impossible, however, that 6p22.3 is driven by more than one oncogene, as it was already demonstrated, e.g. for MDM2 and CDK4 at 12q13-15 **(41, 80)**: among the

questionable genes at 6p22.3, E2F3 and NM_017774 showed highest amplification levels (see **figure 10**).

1.7 CDKAL1 (=NM_017774): a gene with unknown function

The term 'CDKAL1', fully 'Cyclin dependent kinase 5 regulatory subunit associated protein1-*like 1*', suggests a reasonable amount of similarity to the 'CDK5 regulatory subunit associated protein1' (CDK5RAP1). According to 'NCBI Entrez Nucleotide' search engine, the protein encoded by the mRNA sequence of NM_017774 owns specific regions known to have either 'iron ion binding capacity' or 'catalytic activity', respectively. Other functional domains are not yet known. About the actual function of the protein nothing is known.

mRNA^{NM_017774}: .....2642 bp
.....17 exons
protein^{NM_017774}: .....579 aa
.....65 kDa

1.8 Transcriptionfactor E2F3 (=NM_001949)

E2F3 is a transcriptionfactor that binds DNA cooperatively with its associated subunit (DP) through the E2 recognition site, 5'-TTTCGCGC- 3' found in the promoter region of a number of genes whose products are involved in cell cycle regulation or in DNA replication. E2F3 belongs to a family of cell cycle regulatory transcription factors (E2F's; depicted in **figure 11**) that are controlled by the retinoblastoma tumour suppressors (**page 30**).

E2F's are also targets of the transforming proteins of small DNA tumour viruses. The origin of the name goes back to 1987, when a promoter-specific factor was discovered that was regulated by an inducible promoter of the adenoviral early region 1A (=E1A) that is stimulating five viral promoters during the early phase of the viral infection (**53**), in order to direct host cells towards S-phase of the cell cycle. The newly found factor was termed **E**arly **2** **F**actor (=E2F) **(81, 82)**. After the discoveries of several other E2F-family members the complex picture of a functionally heterogeneous gene family became apparent **(5, 83)**: E2F1, E2F2 and E2F3 act as promoters of the G1/S-Phase induction, E2F4, E2F5 and E2F6 are generally regarded either as weak transcriptional activators or as transcriptional repressors.

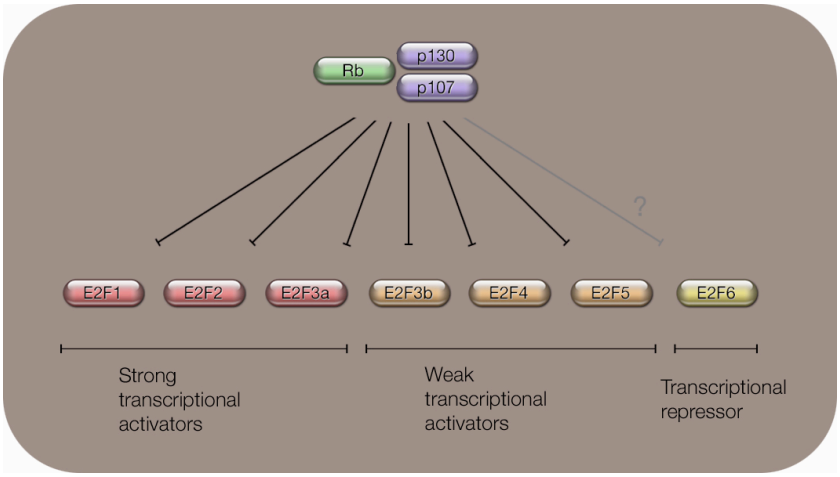

Figure 11: The mammalian genome encodes at least six different E2F transcriptionfactor-proteins, termed E2F1-6. In the mouse (*M. musculus*) the E2F-family can be subdivided according to individual transcription properties and interaction with different pocket-binding proteins. E2F1-E2F3a are potent transcriptional activators and are inactivated by Rb. E2F3b-E2F5 are relatively weak transcriptional activators that are inactivated by p107 and p130 (although E2F4 will interact with all three pocket proteins). E2F6 acts as a transcriptional repressor but does perhaps not associate with pocket-binding proteins *(acc. to Classon & Harlow, Nature 2002; modified)*.

E2F proteins contain several evolutionarily conserved domains. These include a DNA binding domain, a dimerization domain, which determines interaction with the differentiation regulated transcription factor proteins (DP), a 'transactivation' domain enriched in acidic amino acids, and a pocket protein-binding domain. E2F3, along with two other family members (E2F1 and E2F2), have an additional cyclin A binding domain **(84)**, enabling tight control over the expression of these genes during the cell-cycle **(5)**.

mRNA^{E2F3}: .....4744 bp
.....7 exons
protein ^{E2F3}: .....465 aa
.....58 kDa

In the mouse (*M. musculus*), two different variants of E2f3 were found **(4, 85)**. Complex transcriptional regulatory mechanisms control the expression of the E2f3a/b locus **(5)**. The more recently detected E2f3b is missing 101 N-terminal amino acids relative to E2f3a (or full-length E2f3). Absence of the N-terminal region in mouse E2f3b leads to a constant expression of the gene throughout the cell cycle (similar to E2F4 and E2F5). In contrast, E2f3a is only expressed at the G1/S-phase. Both splice variants share the same nuclear localization sequence and the potency to bind Rb (see **figure 12**). This suggests that mouse E2f3a and E2f3b have opposing roles in cell cycle control **(85)**. However, the existence of distinct splice variants has not yet been reported for human E2F3.

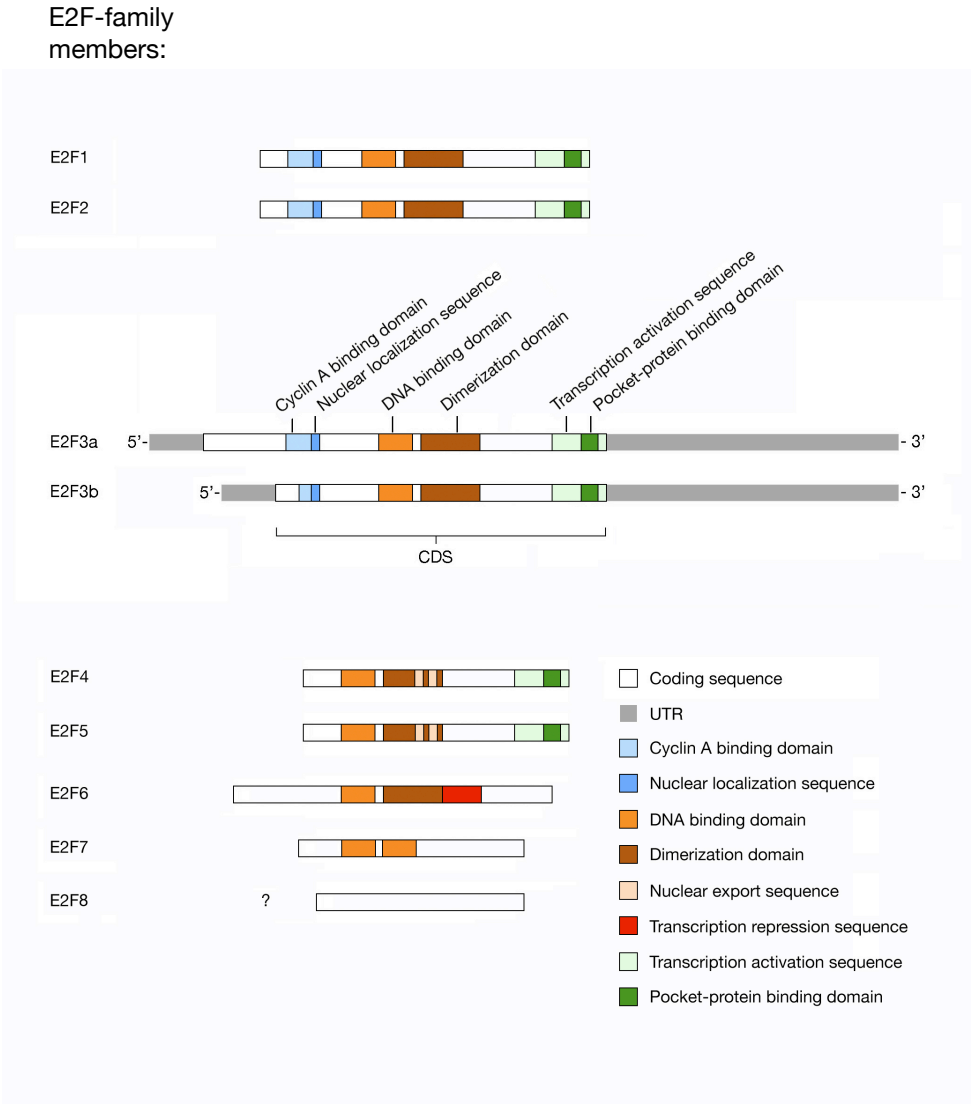

Figure 12: In the mouse (*M. musculus*) two structurally very similar but functionally distinct E2F3 homologs are reported. E2f3b differs structurally from E2f3a by the absence of a short DNA sequence in the 5'-flanking region of the gene, which is assumed to function as a cyclin A binding site **(3-5)**. This difference is said to originate from distinct transcriptional start sites **(5)**, rather than from alternative splicing. According to *Hu & Cress 2000 and 2002, Leone et al., 2000a, Leone et al., 2000b, Adams et al., 2000, Dimova & Dyson 2005.*

BOX1: Role of Rb and E2F's

The synthesis of DNA is a tightly controlled, highly coordinated process. Delays in progression through S-phase as a consequence of DNA damage or insufficient availability of protein or DNA precursors frequently result in cell death, chromosomal abnormalities or mutations. It is not surprising that many mutations in tumour cells are found in genes that are directly involved in cell cycle regulation. To prevent premature entry into the division cycle, mammalian cells have developed elaborate control mechanisms to delay cell cycle progression if DNA damage has has been detected, in order to provide necessary time for the cell to repair. The most significant restriction point occurs in late G1, approximately 4 hours prior to the cell's entry into S-phase. It represents the final checkpoint after which the cell is irreversibly programmed to begin DNA synthesis. This in turn suggests, that the most dangerous DNA alterations occur in cells damaged in late G1 and early S-phase after the restriction point has been passed.

In healthy cells cyclin D is synthesized as a response to mitogen stimulation. Free cyclin D binds to cyclin dependent kinases CDK4 and CDK6. Activated cyclin D/CDK-complexes inactivate Rb by multiple phosphorylations at its C-terminal (see **figure 13**). Inactivation of Rb liberates transcription factors of the E2F-family, which activate genes that are directly involved in G1/S-transition and DNA-replication. In late G1- and during S-, G2-, and M-phase other cyclin/CDK-complexes are formed to maintain the phosphorylated state of Rb unless cytokinesis is completed. If a cell suffers from DNA damage, p53 -the 'guardian of the genome' –induces expression of a series of CDK-inhibitors, including p21, to block Rb phosphorylation by cyclin/CDK-complexes. Under normal circumstances, this cell-cycle arrest is not released before the cell has successfully repaired all damaged DNA-sequences. If this cannot be achieved, p53 will induce apoptosis.

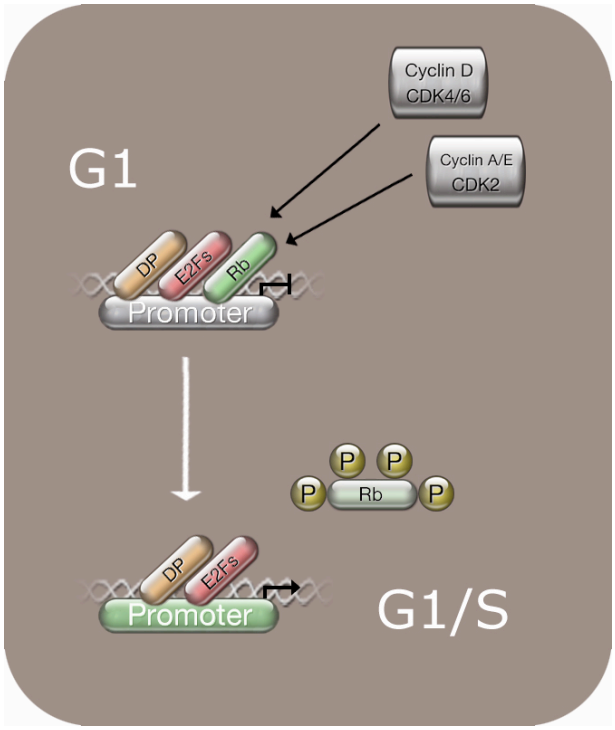

Figure 13:
G1/S-phase induction is mediated via transcription factors of the E2F-family. Hypophosphorylated Rb is believed to bind E2F's and their associated DP-subunits, inducing recruitment of chromatin remodelling complexes to the promoter regions. Phosphorylation of Rb by CDK4/cyclin D and CDK2/cyclin E inactivates Rb and induces the protein to dissociate from E2F/DP-silencing-complexes. This results in activation of E2F-responsive target genes: e.g. cyclin A, -E, -D, DNA polymerase, thymidin kinase, and cdc6.

1.9 Aim of the present study

Bladder cancer contributes significantly to the overall human cancer burden. While over 300'000 individuals are confronted with this disease every year, more than 130'000 patients die annually due to the consequences. Gene amplifications that are frequently detected in malignant tumours, are believed to harbour oncogenes, driving the amplifications.

Previous studies have shown that 6p22 is one of the most frequently amplified regions in urinary bladder cancer. In 2000 *Cancer Research* reported the successful delineation of the chromosome 6p-amplicon, giving rise to intensive investigations aimed at the identification of the responsible oncogene **(72)**. During the following years, several genes could be excluded from further analysis due to low-level or absent expression in 6p22-amplified bladder cancer cell lines. These findings make it unlikely that such genes have an oncogenic role in bladder cancer. In contrast, certain genes earned increasing attention, but it was impossible to conclusively identify the responsible target gene, yet. However, most experts would agree that two relevant genes remain as most likely candidates, the transcriptionfactor E2F3 and a gene of unknown function (NM_017774).

This study was designed to identify the gene (or the genes) at 6p22.3 that is/are responsible for frequent amplification of the genomic region in human bladder cancer. The specific aims included:

- Determination of the amplification frequency of NM_017774 by FISH analysis, by utilizing the same bladder cancer TMA as previously used for E2F3 (includes 2317 bladder tumour samples).

- Evaluation of possible associations between NM_017774 with invasive and high-grade tumour phenotype, and patient prognosis.

- Comparison of E2F3 and NM_017774 amplification frequencies, in order to detect particular tumours that amplify only one gene. Such tumours should be subject to more detailed studies performed by Large-Section FISH, resulting in a precise case-by-case evaluation.

- Quantification and comparison of gene expression levels for E2F3 and NM_017774 in bladder cancer cell lines with and without amplification of 6p22.3.

- Establishment of functional approach in order to determine if E2F3 or NM_017774 is the main amplification target, or if both genes might contribute jointly to the aggressive features of 6p22.3 amplified bladder tumours. This analysis might provide also supplementary information pointing at a presumptive function of NM_017774.

This work is intended to advance our knowledge of the genes and mechanisms involved in human bladder cancer development. Thereby the results of this thesis will hopefully contribute to design better diagnostic and therapeutic procedures to finally reduce mortality rates of the disease in the future.

2 MATERIALS & METHODS

2.1 Tissue collection studied

Three pre-existing TMAs were utilized in this study. The first one is a bladder cancer prognosis array composed of 2317 formalin-fixed paraffin embedded tissues (see **Figure 14**), which was described in detail including histological and clinical data **(75)**. The second is a Multi Tumour Array (MTA) composed of 4788 tissue samples including 3670 primary tumours from 128 different tumour categories. The tissue samples were derived from the following organs or anatomical sites: fetus and placenta, brain, salivary glands, oral cavity, esophagus, stomach, small intestine, colon, appendix, anus, gallbladder, pancreas, liver, larynx, lung, kidney, urinary bladder, prostate, testis, ovary, uterus, vagina and vulva, mammary gland, adrenal gland, thyroid gland, parathyroid gland, thymus, nodal and lymphatic tissue, skin, and soft tissue **(86)**. The third TMA, used in this study, was a cell line TMA containing 18 bladder cell lines which has also been described in detail before **(41)**. All slides of all tumours were reviewed by one pathologist (GS). Tumour stage and grade were defined according to UICC and WHO **(87, 88)**.

2.2 Fluorescence in situ hybridization (FISH)

FISH analysis of the cell line TMA and the bladder cancer TMA was performed with digoxigenated BAC (NM_017774: BAC RP3444C7, RZPD, Berlin, Germany) and PAC (E2F3: PAC dJ177P22, Sanger Centre, UK) probes containing the target genes and a Spectrum Red-labeled chromosome 6 centromeric probe (CEP6) as a reference (Vysis, Downers Grove, IL). Hybridization and posthybridization washes were according to the 'LSI procedure' (Vysis). Amplification was defined as presence (in $\geq$ 5% of tumour cells) of at least 3 times as many target gene specific signals than centromere 6 signals.

For the MTA, two different digoxigenated BAC probes were used, spanning the predicted genomic localizations for E2F3 and NM_017774 (RP3498I24; RP3348I23; RZPD, Berlin, Germany). To accelerate analysis, FISH signals were not scored in a cell-by-cell manner but estimated for each tissue spot by an experienced technician (HN).

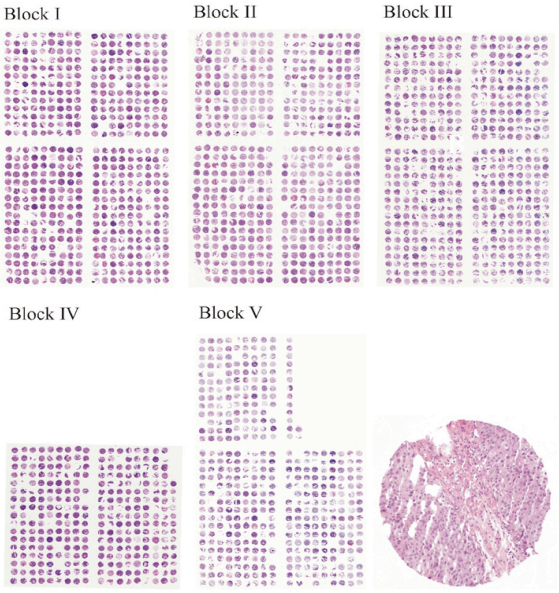

Figure 14: Bladder cancer TMA. Overview of the 5 array blocks, containing 2317 samples. A single tissue-spot, measuring 0.6mm in diameter, is shown at higher magnification. Pictures are courtesy of *Prof. Dr. Guido Sauter*.

2.3 Cell lines and culturing conditions

Cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown under standard cell culturing conditions in Optimem cell culture medium (Invitrogen, Carlsbad, CA), supplemented with 1% penicillin/streptomycin (Amimed, Basel, Switzerland) and 10% FCS (Amimed) at 37° C/ 5% CO_2. Trypsin-EDTA (Amimed) was used as a transferring reagent. The following cell lines were utilized in our experiments: HTB-1 (J82), HTB-2 (RT4), HTB-3 (SCaBER), HTB-4 (T24), HTB-5 (TCC SUP), HTB-9 (5637), CRL-1472 (HT-1376), CRL-2169 (SW 780), CRL-7588 (Hs 853.T), CRL-7882 (Hs 769.T), CRL-7930, RT-112, RT-112 D21, HB-CLS-1, HB-CLS-439, EJ28, BFTC-905 and Ku-1919.

2.4 Northern Blot

Poly-A^+ RNA was isolated from an equal amount of total RNA utilizing Qiagen (Hilden, Germany) Oligotex purification kit. 2 µg poly-A^+ RNA of each sample was processed for Northern blot analysis as described by **(72)**. The house-keeping gene G3PDH was utilized as a control. **Table 3** shows an overview of the hybridisation probes used.

CDKAL1 (NM_017774)	Primer sequences	Method	Probe	Probe
Gene size = 2642 bp; 17 exons			Size (bp)	localization
Primer_F1	5'-CAGGACTACCTTAAGGGACTGA-3'	N-Blot		
Primer_R1	5'-TTTCCATGAGTACGCTGTCG-3'	N-Blot	562	spans exon 6-11
Primer_F2	5'-TATGCCTTCTGCATCCTGTG-3'	real-time PCR		
Primer_R2	5'-AACCCCATGTTCGTATCCAA-3'	real-time PCR	218	spans exon 2-3
siRNA_1	5'-CAGCAGATAGATCGTGTGGTA-3'	real-time PCR	-	inside exon 7
siRNA_2	5'-TGGAATTGGTATACTAAGCAA-3'	real-time PCR	-	inside exon 17

E2F3 (NM_001949)	Primer sequences	Method	Probe	Probe
Gene size = 4744 bp; 7 exons			Size (bp)	localization
Primer_F	5'-GATGGGGTCAGATGGAGAGA-3'	real-time PCR		
Primer_R	5'-GAGACACCCTGGCATTGTTT-3'	real-time PCR	211	inside exon 7
siRNA_1	5'-TTGCGTTACTTTAAGTACTAA-3'	real-time PCR	-	inside exon 7
siRNA_2	5'-TTGGGAGTAGGCAAACTACTA-3'	real-time PCR	-	inside exon 7

G3PDH (NM_002046)	Primer sequences	Method	Probe	Probe
Gene size = 1310 bp; 9 exons			Size (bp)	localization
Primer_F	5'-GAAATCCCATCACCATCTTCC-3'	N-Blot; real-time PCR		
Primer_R	5'-CAGAGATGATGACCCTTTTGG-3'	N-Blot; real-time PCR	156	spans exon 4-6

Table 3: RNAi-duplexes and primer sequences used for gene expression analysis.

2.5 RNA interference

RNA interference specific knock-down of target genes was achieved according to the instructions of Dykxhoorn and colleagues **(89).** The localizations of siRNA-duplexes, and corresponding primers for real-time PCR, are visualized in **figure 15** (for precise RNAi- and primer-sequences see additionally **table 3**). E2F3-specific- and NM_017774-specific-, as well as unspecific RNAi (nonsense-RNAi; control) were purchased from Qiagen. Lipofectamine and Oligofectamine (Invitrogen) were used as transfection reagents according to the manufacturers protocols. Transfection conditions including the number of cells plated and the cells : RNAi : lipid-carrier ratio for efficient transfection were optimised for each cell line in separate experiments. 24h prior to transfection cells were serum starved and then stimulated to re-enter the cell

cycle by the re-addition of serum. Cells were harvested at regular intervals to determine the proliferation rate. At the same time RNA- and protein extractions were performed as described below. Results were normalized against the nonsense-RNAi control.

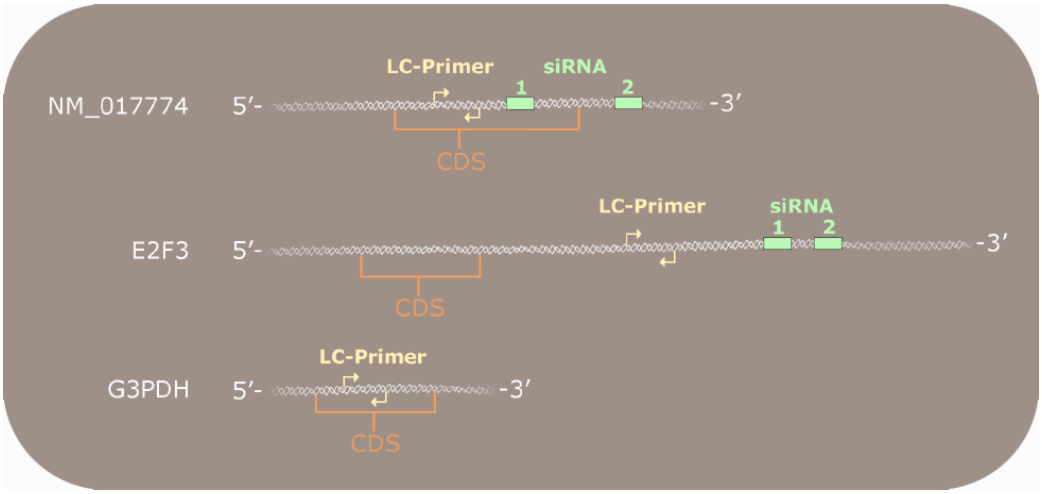

Figure 15: Locations of RNAi-sequences and primers, as employed in RT-PCR analysis.

2.6 Growth curves

To monitor the effect of gene silencing on tumour cell proliferation, growth curves were calculated from RNAi-treated and -untreated cell cultures. Replicate cultures were grown in parallel allowing for repeated cell harvesting and counting in 24h intervals. Cell counting was performed using a 'Neubauer' counting chamber. Standard counting procedures were followed to determine cell quantity **(90)**.

2.7 RNA extraction, cDNA synthesis and Quantitative real-time PCR

RNA isolation was carried out according to manufacturer's specifications using DNase I system in combination with the RNeasy kit (Qiagen, Hilden, Germany). RNA concentration was determined with a spectrophotometer. For each cell line 250 ng total RNA was used as starting material for cDNA synthesis combined with oligo-dT (Roche, Basel, Switzerland) as primer. Real-time PCR was performed in duplicates in 20 µl reactions containing: 2 µl cDNA template (from 1:2 dilutions of cDNA synthesis reaction), 10 µl FastStart SYBR® Green I PCR Master Mix (Roche), $MgCl_2$ as well as forward- and reverse primer mix (10 mM each). Thermal cycling conditions for the

LightCycler Instrument (Roche) were: one cycle at 95° C for 10 min at steps of 20° C/sec (activation), 40 cycles at 95° C for 15 sec at 20° C/sec, 55° C for 10 sec at 20° C/sec and 72° C for 10 sec at 5° C/sec (amplification) and one additional cycle at 95° C for 1 sec at 20° C/sec, 65° C for 15 sec at 20° C/sec and 99° C for 1 sec at 0.05° C/sec (melting). Relative levels of expression were determined using the $2^{-\delta\delta CT}$ method as described by **(91)**.

2.8 Western Blot Analysis

Protein was extracted from cell lines HTB-5, HTB-9, CRL-1472, CRL-7882 and RT-112 as described by Leone et al. (1998). 10 µg protein of each sample was subjected to SDS-PAGE on 10% polyacrylamide gels (Invitrogen) according to the manufacturers protocol. Blots were incubated with mouse monoclonal E2F3 Ab-4 primary antibody (1:1000) (Lab Vision, Fremont, CA) followed by incubation with goat anti-mouse IgG secondary antibody (1:2000) (Fc, AP127P; Juro Supply AG, Lucerne, Switzerland). Finally, blots were processed with the ECL system (Amersham Pharmacia Biotech, Duebendorf, Switzerland) and exposed to Kodak AR film (Stuttgart, Germany).

2.9 Statistics

All tissue samples on the TMA were utilized for comparisons of amplification and overexpression of NM_017774. Only the first biopsy was used for further statistical analyses in patients having more than one tumour on the TMA, in analogy to E2F3 **(75)**. Contingency table analysis and Chi-square tests were applied to study the relationship between histology tumour type, grade, stage, and target gene amplification. Student's *t*-tests were employed to examine the associations of the Ki67 LI with target gene amplification. ANOVA was utilized to determine the parameters with greatest influence on tumour cell proliferation. Survival curves were plotted according to the Kaplan-Meier method and analysed for statistical differences using a log rank test. Finally, contingency table analysis and chi-square tests were applied to rate differences in E2F3 and NM_017774 expression levels in different cell lines and under silenced and non-silenced conditions. Analysis of variance (ANOVA) tests were employed to estimate growth differences between RNAi treated and untreated samples.

3 RESULTS

3.1 Gene amplification

3.1.1 Comparison of NM_017774- and E2F3 amplification frequencies in human bladder cancers

Fluorescence in situ hybridisation (FISH) analysis was performed to compare the amplification frequencies of NM_017774 and E2F3. FISH analysis for E2F3 had been performed before **(75)** but was repeated for this study. This was necessary because FISH signals were not counted cell by cell but estimated for each tissue spot. In this case it is critical that one experienced person (HN) scores all tissue spots in a short period of time to avoid a scoring bias that might occur if different persons perform the scoring or results from different studies are compared. Absolute number of interpretable samples and overall frequency of E2F3-amplifications, were slightly different from our previous analysis. However, all statistical associations established in our recent study **(75)** were also found in the current analysis.

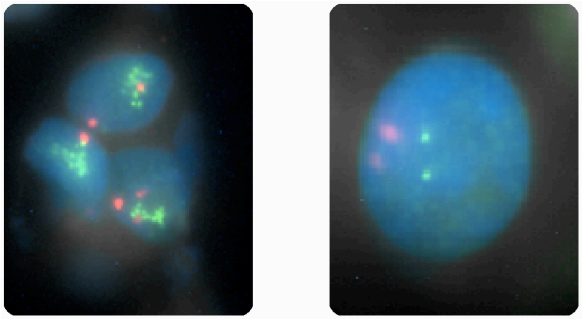

Figure 16: Tumour samples with (left) and without (right) prevalent 6p22.3 amplification.

FISH was successful for both, NM_017774 and E2F3, in 893 of 2317 tumour samples (38.7%). Analysis failures were either due to missing tissue spots or lack of FISH signals. After an initial analysis, amplification frequencies were 9.8% for NM_017774 and 11.4% for E2F3. A subset of 34 tumours (3.8%) could be identified, exhibiting amplification of only one candidate target gene (see **table 4a**). The following case-by-case comparison revealed, that 15 samples (1.7%) were classical borderline artefacts, i.e. where the criterion for the presence of an amplification is marginally fulfilled with respect to E2F3 but is in contrast marginally missed for NM_017774, or vice versa.

Since our study aimed in an as precise comparison of the amplification pattern as possible, the yet remaining 19 tumours (2.1%) with discrepant scores were carefully

re-examined, using conventional large tissue sections. In two cases large sections could not be examined because of insufficient or damaged residual tumour tissue. However, re-analysis was successful for 17 of 19 tumours, and showed that gene copy numbers were in complete agreement for E2F3 and NM_017774 (see **table 4b**). The initially observed discrepancies were either attributable to technical artefacts (n=9), or scoring errors (n=8). In summary, co-amplification of E2F3 and NM_017774, was found in all 6p22.3 amplified tumours (11.6%). As a consequence, amplification of NM_017774 is identically associated with invasive and high-grade phenotype, and patient prognosis as already published for E2F3.

Table 4a: *Initial FISH-Analysis for E2F3 and NM_017774; PT=Primary tumour*

sample	stage/grade	gene copy number			ratio		initial score
		centromer 6	E2F3	NM_017774	E2F3	NM_017774	
PT1	PT1/G3	6	15	20	2.5	3.3	borderline
PT2	PT1/G3	4	10	12	2.5	3.0	borderline
PT3	pT1/G3	2-4	8	15	2.7	5.0	borderline
PT4	pT2-4/G2	2-4	8	10	2.7	3.3	borderline
PT5	pTa/G2	1-2	4	5	2.7	3.3	borderline
PT6	pT2-4/G3	2-5	10	15	2.9	4.3	borderline
PT7	pT2-4/G3	2-5	10	12	2.9	3.4	borderline
PT8	PT1/G3	2	6	5	3.0	2.5	borderline
PT9	PT1/G2	1	3	2	3.0	2.0	borderline
PT10	PT1/G3	4-6	15	10	3.0	2.0	borderline
PT11	pT2-4/G3	2-5	12	10	3.4	2.9	borderline
PT12	PT1/G3	1-2	6	4	4.0	2.7	borderline
PT13	pTa/G3	2	8	5	4.0	2.5	borderline
PT14	pT2-4/G3	1	4	2	4.0	2.0	borderline
PT15	pT2-4/G3	1	4	2	4.0	2.0	borderline
PT16	pT1/G3	2-4	10	2	3.3	0.7	discrepant
PT17	pT1/G3	2	10	2	5.0	1.0	discrepant
PT18	pT1/G3	2-5	3	13	0.9	3.7	discrepant
PT19	pT2-4/G3	2	12	2	6.0	1.0	discrepant
PT20	pT1/G3	4	12	2	3.0	0.5	discrepant
PT21	pT2-4/G3	2-5	6	15	1.7	4.3	discrepant
PT22	pTa/G3	2	15	2	7.5	1.0	discrepant
PT23	pT1/G3	2-4	10	2	3.3	0.7	discrepant

PT24	pT2-4/G3	2-3	10	2	4.0	0.8	discrepant
PT25	pT2-4/G3	2-3	2	20	0.8	8.0	discrepant
PT26	pT2-4/G3	2-4	10	2	3.3	0.7	discrepant
PT27	pT1/G3	2-3	2	35	0.8	14.0	discrepant
PT28	pT2-4/G3	2-4	15	2	5.0	0.7	discrepant
PT29	pT1/G3	2	15	2	7.5	1.0	discrepant
PT30	pT1/G3	1-2	2	15	1.3	10.0	discrepant
PT31	pT1/G3	2-4	4	10	1.3	3.3	discrepant
PT32	pT1/G3	1-2	2	30	1.3	20.0	discrepant
PT33	pT2-4/G3	2	20	2	10.0	1.0	discrepant
PT34	pT1/G3	1	20	4	20.0	4.0	discrepant

Table 4b: *Large section FISH-Analysis for E2F3 and NM_017774.*

sample	stage/grade	gene copy number			ratio		final score
		centromer 6	E2F3	NM_017774	E2F3	NM_017774	
PT16	pT1/G3	2-3	2-3	2-3	1.0	1.0	normal
PT17	pT1/G3	2-4	2-4	2-4	1.0	1.0	normal
PT18	pT1/G3	2-4	2-4	2-4	1.0	1.0	normal
PT19	pT2-/G3	2-5	2-8	2-6	1.4	1.1	gain
PT20	pT1/G3	4-8	4-12	4-12	1.3	1.3	gain
PT21	pT2-/G3	2-6	2-10	2-6	1.5	1.5	gain
PT22	pTa/G3	2-10	10-20	10-20	2.5	2.5	gain
PT23	pT1/G3	2-4	6-10	6-10	2.7	2.7	gain
PT24	pT2-/G3	2-4	10	10	3.3	3.3	co-amp
PT25	pT2-/G3	4	10-20	20	3.8	5.0	co-amp
PT26	pT2-/G3	2	10	10	5.0	5.0	co-amp
PT27	pT1/G3	2-4	10-20	10-20	5.0	5.0	co-amp
PT28	pT2-/G3	2-4	10-20	10-20	5.0	5.0	co-amp
PT29	pT1/G3	2-4	10-30	10-30	6.7	6.7	co-amp
PT30	pT1/G3	1-2	10-20	10-20	10.0	10.0	co-amp
PT31	pT1/G3	2-4	30	30	10.0	10.0	co-amp
PT32	pT2-/G3	2-6	50	50	16.7	16.7	co-amp
PT33	pT2-/G3	tissue *	-	-	-	-	-
PT34	pT1/G3	tissue †	-	-	-	-	-

tissue * = insufficient tissue leftover

tissue † = tissue damage

3.1.2 Amplification of NM_017774 and E2F3 in bladder cancer cell lines

To once for all determine the 6p22.3-amplification status of bladder cancer cell lines potentially used for further investigations, we performed FISH in 18 cell lines: HTB-1, HTB-2, HTB-3, HTB-4, HTB-5, HTB-9, CRL-1472, CRL-2169, CRL-7588, CRL7882, CRL-7930, KU-1919, BFTC-905, EJ-28, RT-112, RT-112 D21, HB-CLS-439 and HB-CLS-1. Results, including precise gene copy numbers are displayed (see **figure 17**). 4 out of 18 showed amplification of 6p22.3 (22.3%). E2F3 and NM_017774 could only be found co-amplified. Highest copy numbers were present in HTB-9, HTB-5 and CRL-1472. HB-CLS-439 revealed moderately increased target gene copy numbers.

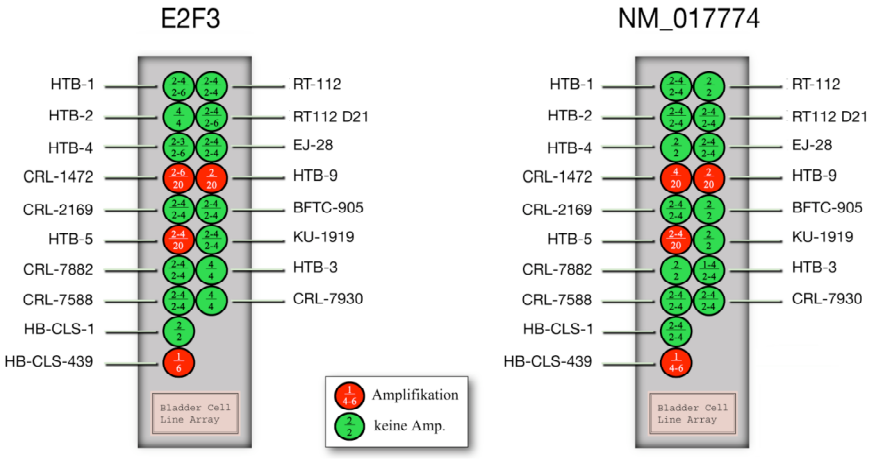

Figure 17: FISH performed in 18 bladder cancer cell lines showed complete correlation according to the amplification status of both target genes (E2F3, NM_017774). Furthermore, observed gene copy number increase was also similar for both genes.

3.1.3 Detection of E2F3-amplification in other cancer types

The E2F3 copy number was determined *in situ* in 4788 tissue specimens. The tissue samples included 3670 primary tumours (from 128 different tumour categories), 720 metastases (from 31 different tumour categories), and 359 normal tissues (from 40 different tissue categories).

Successful hybridizations were observed in 2296 of the 4788 specimens (48%). E2F3 amplification was found in 14 bladder cancer specimens and, in addition, in two seperate cases of breast cancer (one breast cancer primary tumour and one breast cancer metastasis; see **table 5**). No other cancer types showed E2F3 amplification.

Table 5 *Tumour categories with prevalent E2F3-amplification*

Tumour categories	Interpretable (n=2296)	E2F3-amplified (n=16)
Breast cancer primary tumour	43	1
Breast cancer metastasis	181	1
Bladder cancer muscle invasive (pT2-4)	70	13
Bladder cancer non-invasive (pTaG2)	42	1

3.2 Gene expression analysis

3.2.1 Simultaneous quantification of target gene expression (by Real-time PCR)

The influence of 6p22.3-amplification on the expression of NM_017774 and E2F3 was quantified by real-time PCR. Results are summarized in **figure 18**.

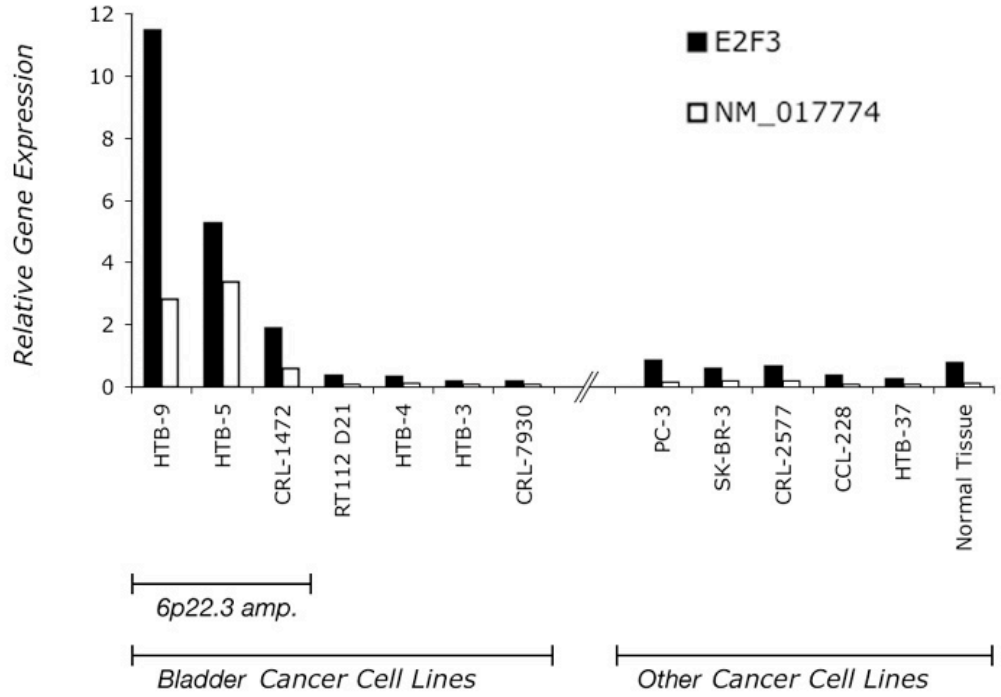

Figure 18: Relative gene expression of E2F3 and NM_017774 in various cancer cell lines with and without 6p22.3 amplification. E2F3 revealed on average 4.25-fold higher expression levels than NM_017774. Both genes were markedly upregulated in amplified cell lines. The data was obtained by means of SYBR® Green real-time PCR (Roche). All samples were normalized against G3PDH, utilizing the $2^{-\Delta\Delta CT}$ method.

In total, 7 bladder cancer cell lines, 3 colon cancer cancer cell lines, 1 prostate cancer cell line, and 1 sample of normal bone marrow were tested. Amplification of 6p22.3 was strongly associated with increased expression of both E2F3 and NM_017774. On average, expression of the target genes was more than 10-fold higher in amplified cancer cells as compared to non-amplified cells. Besides, E2F3 was stronger expressed than NM_017774 in all cell lines analysed (average difference 4.25-fold; p=0.0009).

3.2.2 RNA expression of NM_017774 (by Northern-blot analysis)

Expression of NM_017774 could not be observed by Western blot (see **below**), because no adequate antibody is currently available. Therefore, the expression of NM_017774 was instead determined by Northern blot analysis utilizing 3 amplified (HTB-5, HTB-9 and CRL-1472) and 3 non-amplified (RT-112, RT-112 D21 and CRL-7930) bladder cancer cell lines. Expression of NM_017774 was in perfect correlation with 6p22.3-amplification (see **figure 19**). Highest expression of NM_017774 was found in HTB-5, followed by HTB-9 and CRL-1472.

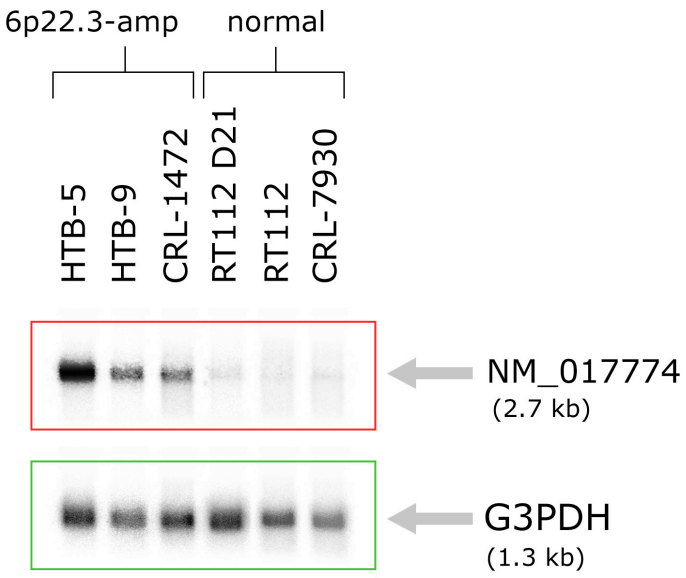

Figure 19: Northern blot analyses of NM_017774 gene expression. Expression of G3PDH is shown as a control. Expression of NM_017774 is markedly increased in bladder cancer cells exerting 6p22.3-amplification.

3.2.3 E2F3 protein expression (Western-blot analysis)

To estimate the influence of gene amplification on protein expression, Western blot analysis was performed with three amplified (HTB-5, HTB-9 and CRL-1472) and two non-amplified bladder tumour cell lines (CRL-7882, RT-112). All three amplified, but none of the non-amplified cell lines, showed strong E2F3 protein expression (**figure 20**).

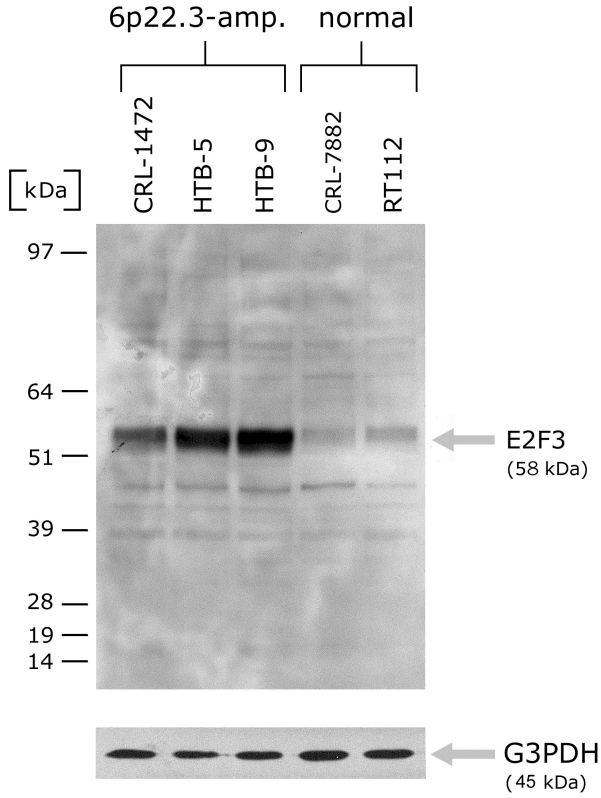

Figure 20: Western blot analysis of E2F3 in 6p22.3-amplified bladder cancer cell lines (CRL-1472, HTB-5, HTB-9) and in non-amplified cell lines (CRL-7882, RT-112). Amplified cell lines show a massive increase of E2F3 protein expression as compared to non-amplified bladder cancer cell lines. Weak non-specific bands are seen in both amplified an non-amplified cell lines.

3.3 Functional Analysis

In the past few years, several discoveries have underlined the importance of small RNAs for a variety of cellular functions. Small interfering RNAs (siRNAs), which are produced endogenously from cleavage of long double-stranded RNA molecules, elicit gene silencing via targeted mRNA destruction as part of the RNA interference (RNAi) pathway. siRNAs are now commonly used as tools to specifically silence target gene expression.

Two 6p22.3 amplified (HTB-5; CRL-1472) and two non-amplified cell lines (CRL-7930; PC-3) were tested for their suitability for subsequent RNAi experiments. The tests served to clarify the following points:

- Choice of a bladder cancer cell line that shows strong silencing of both target genes

- Determination of the most powerful RNAi-duplex

- Selection of a transfection reagent that leads to transfection of approximately 90% of the present cells is highly recommended

SYBR® green real-time PCR (LightCycler, Roche) was employed to measure the effect of RNAi on target gene expression. Gene knock-down rates of at least -46% after 24h were achieved at minimum (see **figure 21)**. Based on this experiment, the non-amplified cell line CRL-7930 (E2F3: 73% knock-down; NM_017774: 59% knock-down) and the amplified cell line HTB-5 (E2F3: 53% knock-down; NM_017774: 50% knock-down) were selected for subsequent experiments, in combination with Lipofectamine as the transfection reagent. As a general rule, cell lines with normal 6p22.3 copy numbers (CRL-7930, PC-3) offered slightly higher knock-down rates than amplified cell lines (CRL-1472 and HTB-5).

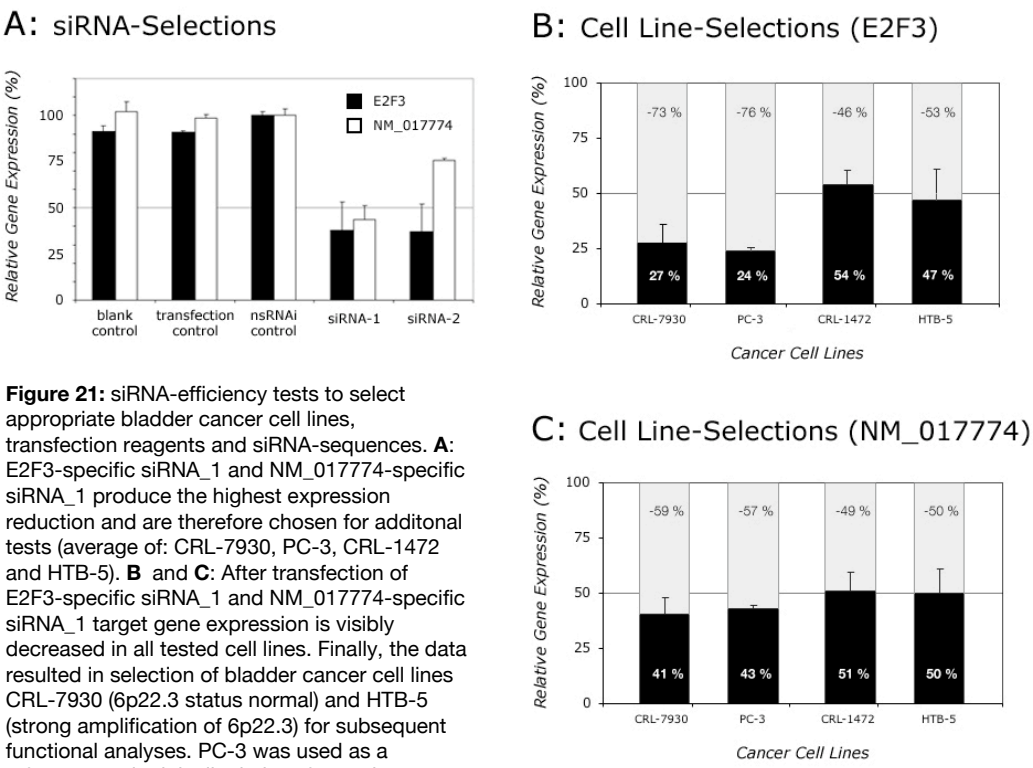

Figure 21: siRNA-efficiency tests to select appropriate bladder cancer cell lines, transfection reagents and siRNA-sequences. **A**: E2F3-specific siRNA_1 and NM_017774-specific siRNA_1 produce the highest expression reduction and are therefore chosen for additonal tests (average of: CRL-7930, PC-3, CRL-1472 and HTB-5). **B** and **C**: After transfection of E2F3-specific siRNA_1 and NM_017774-specific siRNA_1 target gene expression is visibly decreased in all tested cell lines. Finally, the data resulted in selection of bladder cancer cell lines CRL-7930 (6p22.3 status normal) and HTB-5 (strong amplification of 6p22.3) for subsequent functional analyses. PC-3 was used as a reference and originally derives from a bone marrow metastasis of a prostate primary tumour.

3.3.1 Gene knock-down efficiency during proliferation assay

The two selected RNAi-sequences lead to powerful target gene knock-down. The impact was detectable 12h after transfection (see **figure 22**). After one day, target gene expression was already more than halved.

In cell line CRL-7930 mRNA levels decreased until the end of the experiment (day 6). On average, silencing was slightly more efficient for E2F3 (73% decrease of mRNA level; average from days 4-6) than for NM_017774 (59% decrease of mRNA level; average from days 4-6; p=0.0016).

In the amplified cell line HTB-5 the lowest mRNA expression levels were reached between 24h and 48h after transfection. Starting with day 3, target gene expression begun to recover slowly but constantly. No difference between average knock-down of E2F3 (53%) and NM_017774 (50%) was detectable (p=0.4186). Interestingly, combined knock-down did not result in a further decrease of individual mRNA-levels as compared to separate knock-down, in all tested cell lines.

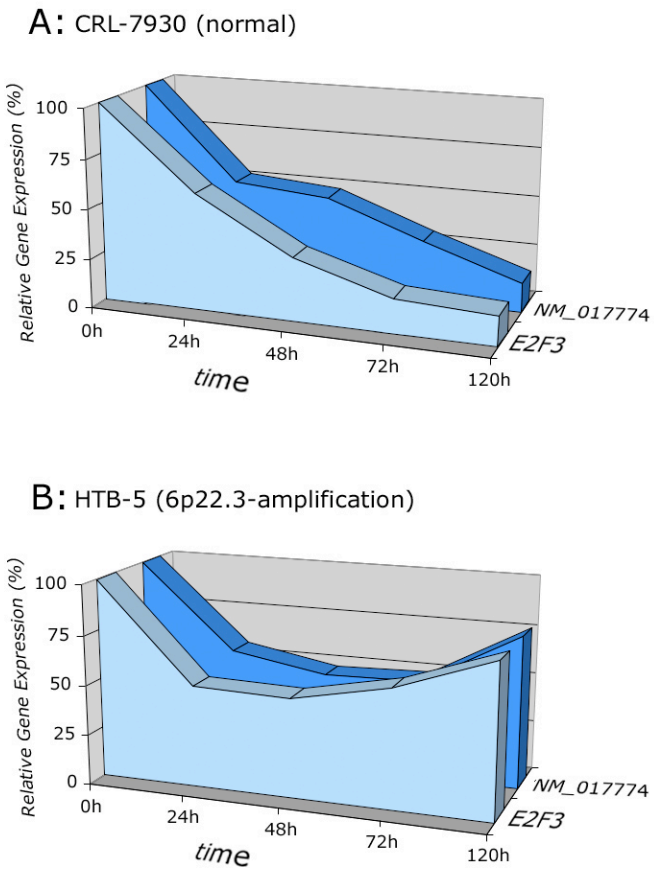

Figure 22: During the entire period of the proliferation assay, the mRNA knock-down was tightly controlled. Powerful reductions in target gene expression were observed during the first 24h after transfection, independently from the utilized bladder cancer cell lines. However, after 48h, differences between the cell lines became visible: Whereas in the non-amplified CRL-7930 cells (**A**) they continued to decrease until the end of the experimental period, the mRNA levels of both target genes began to recover after the first two days in the amplified HTB-5 cells (**B**).

3.3.2 Proliferation assay

The distinctive effects of E2F3-, NM_017774- and combined gene knock-down on cell proliferation were compared against the effect of nonsense-RNAi control and are visualized in **figure 23**. In the non-amplified cell line (CRL-7930) knock-down of E2F3 and NM_017774 resulted each in a pronounced decrease of the cell proliferation rate (average over 6 days: E2F3: -43.1%; NM_017774: -48.7%; maximum E2F3: -48.2% at day 4; maximum NM_017774: -55.4% at day 4). Simultaneous knock-down exerted an even stronger decrease amounting to –57.9% (maximum -69.6%; day 4).

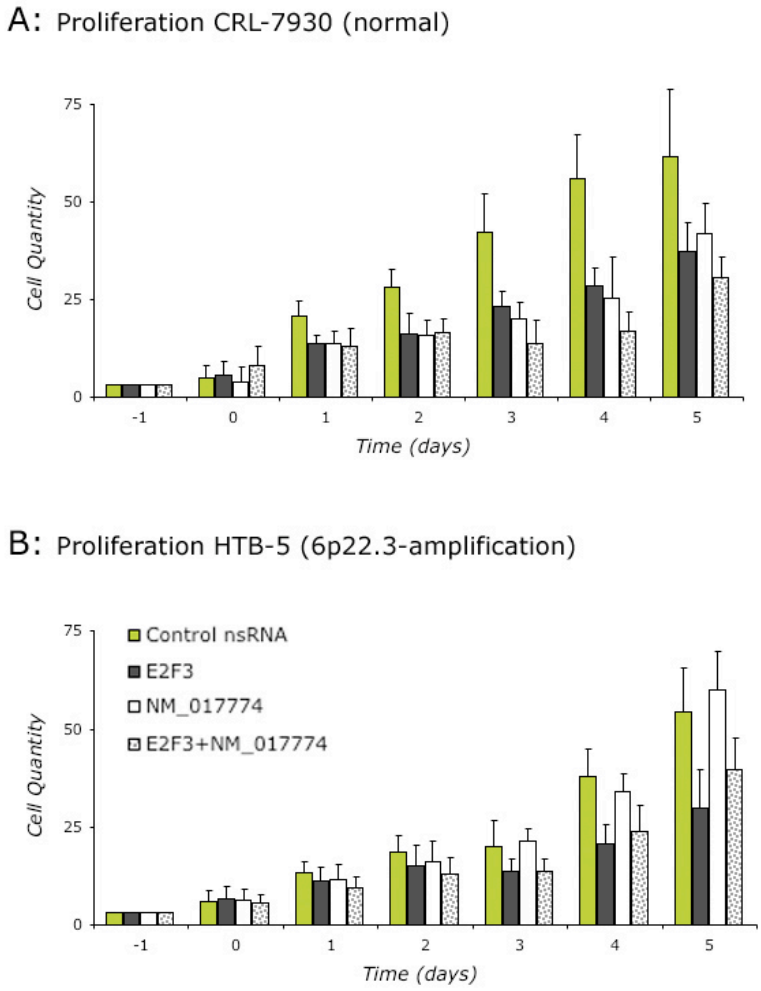

Figure 23: Cell proliferation of bladder cancer cell lines with and without 6p22.3 amplification. Bars illustrate observed differences in cell proliferation rates after gene specific knock-down of potential amplification targets E2F3 and NM_017774. Silencing of E2F3 always markedly decreased cell proliferation (**A**+**B**), whereas silencing of NM_017774 only inhibited non-amplified CRL-7930 cells (**A**) but failed to affect proliferation in amplified HTB-5 cells (**B**). Controls, treated with unspecific RNAi, are shown in green.

In the amplified cell line (HTB-5) knock-down of E2F3 resulted in a comparable decrease of the cell proliferation, as it had been observed in non-amplified cells (average: -36.7%; maximum: -44.7% at day 4). In contrast, knock-down of NM_017774 had no negative influence on cell proliferation in amplified cells (average: +5%; maximum –10.9% at day 4). The combined knock-down (average: -27.7%, max -36.8% at day 4) reached values analogous to E2F3 alone.

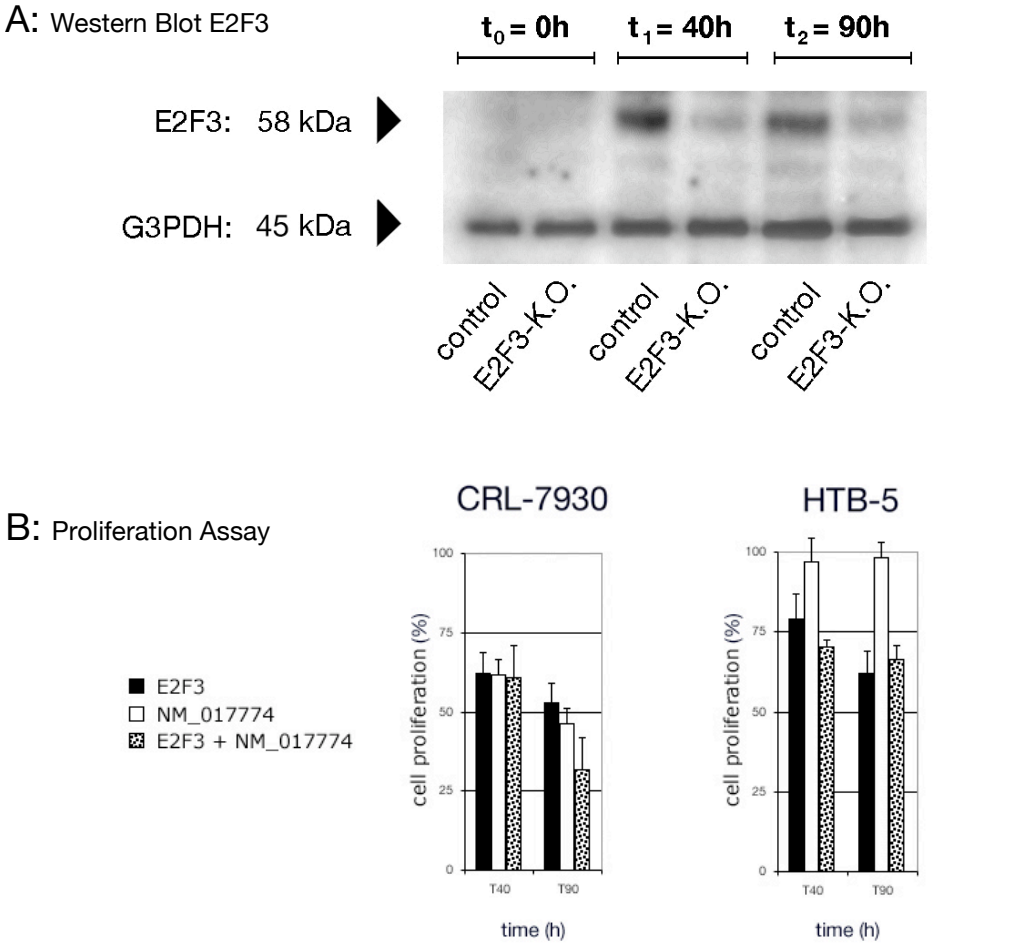

Figure 24: Western blot analysis (**A**) displays the massive reduction of E2F3 expression induced by E2F3-specific gene silencing that lead to the proliferation decrease in 6p22.3-amplified cancer cells (HTB-5). As a comparison, with nsRNAi-treated HTB-5 cells are shown in parallel, and offer normal E2F3 protein concentrations (see control). Protein concentrations are visualized for t_0=0h, t_1=40h and t_2=90h. Corresponding G3PDH-levels can be viewed at the bottom. Inhibition of E2F3 (mRNA; Protein) leads to powerful decrease of cell proliferation in 6p22.3-amplified bladder cancer cell line HTB-5, as well as in the non-amplified control CRL-7930. Results of the proliferation assay (**B**) are additionally provided at the bottom.

The fraction of apoptotic cells was estimated during cell counting (data not shown). There was no obvious difference between control cell cultures treated with transfection

reagent and non-sense RNAi as compared to cell cultures treated with target specific RNAi. The observed differences in cell proliferation between cells with and without E2F3 or NM_017774 knockdown, therefore, are clearly due to the silencing effect and not caused by apoptosis. Besides, we did not observe any changes in cell morphology as a consequence of gene silencing.

The silencing power of E2F3-specific RNAi, resulting in reduced proliferation of HTB-5 cells, was not only confirmed by real-time PCR but also by western blot analysis (**figure 24** gives an informative overview). The silencing power of NM_017774-specific RNAi could not yet be shown, because NM_017774-specific antibodies are currently not available.

3.4 Bioinformatics research: The gene NM_017774 and its possible function

In order to learn more about possible functions of NM_017774, we compared the gene with its next closest relative CDK5RAP1. First, the mRNA sequences of both genes was aligned using the 'Vector NTI'. This program allows similarity searches on mRNA- and/or protein-sequence basis. As a control, we compared both splice variants of the CDK5RAP1. Results are shown in **figure 25**.

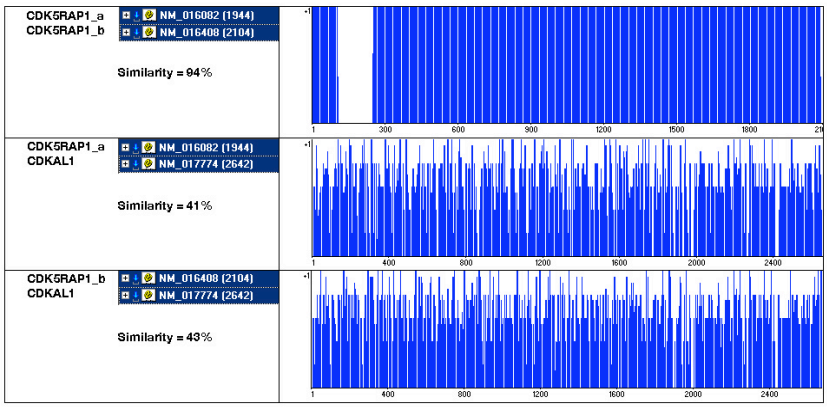

Figure 25: Sequence homology of NM_017774 (=CDKAL1) and both splice variants of CDK5RAP1. Whereas both splice variants turned out to be highly similar except for the additional 5'-region that is only present in the CDK5RAP1b (this region was incorporating 148 bp, or 90 aa, respectively), NM_017774 showed less than 50% similarity to both splice variants.

To collect structural information about the protein product of NM_017774 that would perhaps point at a possible function, we employed CDART ('Conserved Domain Architecture Retrieval Tool'). The analysis revealed that three functional domains are shared with CDK5RAP1, including UPF0004, Radical-SAM, and TRAM. The function of

the UFP0004 domain is unknown, but it is almost always found in conjunction with Radical-SAM and TRAM. Radical-SAM is believed to be involved in radical-based catalysis in a number of previously well-studied but unresolved biochemical pathways, including unusual methylations, isomerization, sulfur insertion, ring formation, anaerobic oxidation and protein radical formation **(92)**. TRAM presumably represents a RNA-binding domain. The TRAM domain is present in several other proteins associated with the translation machinery and may also play a role in the regulation of tRNA modification or translation **(93)**. Alternatively, NM_017774 can be aligned using the 'EXPASy BLAST2 Interface', showing a score of: 24% identity, 46% positives and 14% gaps to both CDK5RAP1 splice variants (www.expasy.org/cgi-bin/blast.pl; reference number Q5QP46).

In summary, although only little is known about the possible function of NM_017774, the presence of particular functional domains in the predicted protein structure link it to the protein translation machinery **(94)**. The most important information found during our investigations is summarized in **table 6**.

		E2F3	CDKAL1	CDK5RAP1
Homolog (NCBI Entrez Homolo Gene)	*H. sapiens*	NM_001949	NM_017774	NM_016082/ NM_016408
	P.troglodytes	LOC471865	LOC462466	LOC458179
	C. familiaris	LOC488239	-	LOC477195
	R. norvegicus	LOC291105	similar to RIKEN cDNA	Cdk5rap1
	M. musculus	E2f3	Cdkal1	Cdk5rap1
	G. gallus	LOC420824; pfam02319)	LOC420823	CDK5RAP1
	X. xenopus	-	-	-
	D. rerio	-	cdkal1	-
	D. melanogaster	-	CG6550	CG6345
	A. gambiae	-	1274919	1280572
	C. elegans	-	radical SAM(1B833)	3H34
	A. thaliana	NP_973611 / NP_973610	At1g72090	At4g36390
	O. sativa	-	OSJNB008...	-
Molecular Structure (NCBI)	MiaB-meth. synth.			
	Elp3-family			
	smart00729			
	MiaB-family			
	Radical SAM			
	pfam01938			
	TRAM			
	UPF0004		pfam00919 related	
	DNA-binding dom.			
	Dimerization dom.			
	Transactivation dom.			
	Tumor sup. ass. dom. (ins. Transac.)			
	Cyclin binding dom.			
	p107/p130 binding dom.	only mouse **E2F3b**		
	pRB binding dom.	**E2F3** and **E2F3a**		
Molecular Function (NCBI)	Iron ion binding		radical SAM	radical SAM
	Catalytic activity		MiaB	MiaB
	neuronal Cdc2-like kinase binding			
	tRNA modifications		no-TRAM (?)	TRAM
	Enzymatic catalysis			
	Methylthiolation		MiaB	MiaB
	Protein binding			
	DNA-binding			
	Transcription factor activity			
	Transcriptional activator activity			
	Transcr. Initiation Pol II promotor			
Molecular Process Involved (NCBI)	DNA replication			
	DNA recombination			
	DNA repair			
	Transcription			
	Translation		MiaB	MiaB
	Reg. of cell cycle			
	Cell proliferation (Repressor)	only mouse **E2F3b**		
	Cell proliferation (Promotor)	**E2F3** and **E2F3a**		
	Neg. reg. of cyclin dep. prot. kin. act.			
	Ribosomal structure			
	Ribosomal biogenesis			
	Reg. of neuronal diff.			
	Brain development			

Legend:

Tram = Translocating chain-associating membrane protein
radical SAM = radical S-adenosyl-L-methionine-related to Elp3
MiaB = 2-methylthioadenine synthetase
UPF0004 = Uncaracterized protein family
pfam = Sequence from protein family database (=protein collection)
Elp = Elongator protein 3

unknown
predicted
strong evidence

Table 6: Predicted homolog, structure, molecular functions and involvement in molecular processes of: NM_017774 and CDK5RAP1. According to information of the *NCBI Research Database 2005*. Scientific evidence increases from light to dark green. The same set of information is additionally provided for E2F3.

4 DISCUSSION

Early mortality has declined in modern human populations throughout the past several 100 years due to improvements in hygiene and nutrition. However, as more people survive into old age, more individuals encounter the diseases associated with old age. As a consequence, particularly the risk of cancer has constantly increased during the last decades, and the ageing of the population in developing countries is pointing to an even further rise in the future.

Bladder cancer contributes significantly to the overall human cancer burden. While over 300'000 individuals are confronted with this disease per year, more than 130'000 patients die due to the consequences. In Western societies the impact of bladder cancer is even somewhat higher, probably as a result of increased life expectancies in combination with modern life-styles, making it the 5th most common malignancy in men in Western societies.

Like in other malignancies, the precise fact why one person develops bladder cancer while others don't, is not yet fully understood. The origin of the disease is now believed to be multifactorial, and several risk factors have been noted. Development of bladder cancer is highly complex and involves various different abnormalities. While it is certain that molecular alterations are required for initiation and progression of the disease, only little is known about the exact nature of these events and the sequence in which they occur.

Most often, the progression of cancer is accompanied by gene amplification, which represents perhaps the most common mechanism of oncogene activation. Gene amplifications that are often detected in tumours are believed to harbour oncogenes that drive the amplifications. More than 30 different chromosomal loci are frequently amplified in bladder cancer. For some of them, the responsible target gene is already identified: CCND1 at 11q13 **(43)**, ERBB2 at 17q21 **(95)**, or MDM2 at 12q13 **(39)**. Evidence for neighbouring oncogenes that undergo co-amplification, followed by co-overexpression has recently been reported at 12q13-q15 (harbouring MDM2, GLI, CDK4, and SAS) **(80).** Presumptive oncogenes for other important amplicons, e.g. 1p36, 4q, 9p, 20q and 6p22, remain undetermined.

Amplification of the chromosomal region 6p22.3 occurs in about 10-20% of human bladder cancers, being one of the most abundant genetic alterations of this tumour type. This fact has lead to the hypothesis, that 6p22.3 must harbour at least one

potential oncogene that is responsible for the high amplification frequency. Intensive investigations aimed in the identification of the target gene, have resulted in the delineation of the chromosome 6p22-amplicon, so far **(72)**. Over the last 5 years, the presence of at least 13 genes inside the 6p22.3 amplicon has been claimed: Q9H1N9, PRL, SOX-4, NM_017774 (=CDKAL1), E2F3, OACT1, ID-4, TFAP2, HMGIY, CCND3, IRF4, HOX12, and PIM1 (see **table 7**).

In a recent study the amplicon could be narrowed down to 1.7 Mb at 6p22.3 **(75)**, and as a consequence the list of presumptive genes could be reduced to: ID-4, OACT1, E2F3, NM_017774, SOX-4 and PRL, and several predicted transcripts. However, verification of individual target gene expression levels exposed weak or absent expression in certain cases, making it very unlikely that such genes play an important role in the development of the disease **(72-74, 76, 79)**. Based on this evidence, 4 local genes were excluded from further studies, including ID-4, OACT1, SOX-4 and PRL.

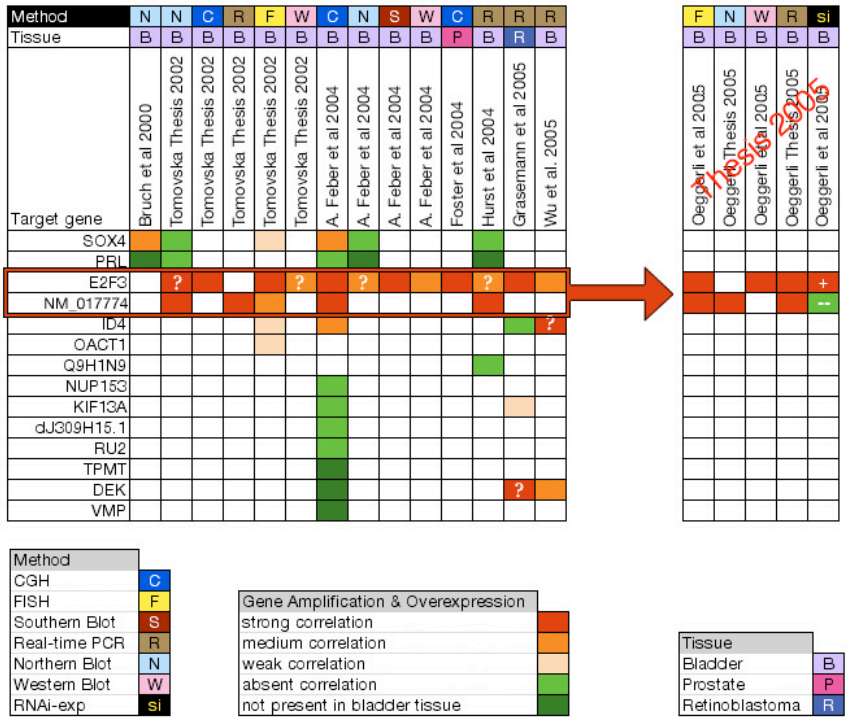

Table 7: Overview of the existent amplification, expression and functional studies aimed in the detection and identification of potential oncogenes localized at 6p22.3. E2F3, as well as its closest neighbouring gene NM_017774 (which is also known as CDKAL1 or FLJ20342) are clearly the most suspected targets to drive the amplification.

In contrast, expression patterns of two other candidate genes, suggests an involvement in 6p22.3-amplification. E2F3 was detected to be strongly amplified and overexpressed in a high percentage of human bladder cancers **(74, 76),** as well as in

some prostate cancers **(79)**. Furthermore, E2F3 was found amplified in 19.8% of invasive bladder tumours, making it the most frequently amplified gene in invasive bladder cancer. Using the same methodology including FISH and TMAs and copy number cut-off levels, lower amplification frequencies were observed for HER-2 (15.3%) and CCND1 (11.1%). Besides 6p22.3 there are no other molecular alterations showing such marked differences between non-invasive (0.7%) and invasive (19.8%) bladder cancers, stressing the importance of the responsible target gene. However, both E2F3 and NM_017774 show comparable amplification frequencies, rendering it impossible to tell which one is the more likely amplification target based on gene copy number information alone.

Therefore, we performed amplification, expression, and functional analyses of E2F3 and its neighbouring gene NM_017774. Our results clearly favour E2F3 as the primary target gene of 6p22.3 amplification in urinary bladder cancer.

First of all, a large tissue microarray comprising more than 2,000 bladder cancer samples was utilized to compare E2F3 and NM_017774 amplification patterns. Although we had analysed E2F3 amplification before **(75),** this analysis was repeated to have the same person scoring both E2F3 and NM_017774. The slightly higher rate of amplified cases in this study (11.6%) as compared to the previous one (7%) was not unexpected because of the higher fraction of analyzable high-grade tumours in the present study. In addition, estimating the number of signals instead of counting may have resulted in some inter-observer variability. Slight inter-observer differences are regularly seen in TMA studies where large numbers of tissues are scored in a short period of time, but have no influence on the significance of findings because of the high statistical power in studies with large patient numbers **(96)**. It was therefore expected, that all statistical associations established in our recent study **(75)** were also found in the current analysis.

After initial analysis, amplification frequencies were 9.8% for NM_017774 and 11.4% for E2F3. Interestingly, not all tumours showed co-amplification of both target genes. A fraction of 34 tumours was identified, exhibiting only amplification of one gene or the other. Since a presumptive oncogene that drives the amplicon is expected to be involved in all tumours with 6p22.3 amplification, the true absence of either E2F3 or NM_017774 in one or more 6p22.3 amplified bladder cancers would make it very unlikely that this particular gene plays an important role in the amplicon. Therefore, the subset of 34 ‘discrepant’ tumours was subject to thorough case-by-case re-evaluation by conventional large tissue sections. This re-evaluation demonstrated, however, that

every tumour with E2F3 amplification had also NM_017774 amplification, and vice versa. The initially observed discrepancies were either due to variable interpretation of borderline findings in low level amplified tumours (15 cases) or counting errors because of low FISH signal intensities, high background, tissue damage and technical artefacts (19 cases). In summary, co-amplification of E2F3 and NM_017774, was found in all 6p22.3 amplified tumours (11.6%). As a consequence, amplification of NM_017774 is identically associated with invasive and high-grade phenotype, and patient prognosis as already published for E2F3 **(75).**

Since FISH analysis could not identify either E2F3 or NM_017774 as the 6p22.3 target gene, we performed RNA- and protein expression analysis as a second step. Because of the superior RNA quality in freshly collected tissues as compared to formalin fixed paraffin embedded samples we analysed RNA levels in amplified versus non-amplified bladder cancer cell lines. We hypothesized that the amplification target gene would show a particularly strong mRNA and protein expression increase.

Highly enriched levels of E2F3 expression have been previously found in 6p22.3 amplified bladder cancer cell lines CRL-1472, HTB-5 and HTB-9 by Northern blot **(76, 97)**. In contrast, only little is known about NM_017774 expression **(74)**. To compensate for the still expected evidence we analysed mRNA expression of NM_017774 by Northern blot analysis. Overexpression of NM_017774 was detected to be in perfect correlation with 6p22.3 amplification. The gene was highly expressed in 6p22.3 amplified cell lines, whereas expression in non-amplified cell lines was almost absent. Highest levels were observed in HTB-5, similarly to recent findings by real-time PCR analysis **(74)**.

To quantitatively investigate target gene expression as a result of 6p22.3 amplification we additionally performed real-time PCR. Expression of E2F3 was on average 4.25-fold stronger than expression of NM_017774 in 'normal' bladder cancer cell lines, without amplification of 6p22.3. Gene amplification had a comparable influence on the expression of both target genes, scaling up individual mRNA levels at least by a factor 10. However, mRNA-increase, as a result of the amplification, was on average slightly higher for NM_017774 than for E2F3. These findings are in line with a previous analysis by **(74)** in cell lines HTB-5, HTB-9, JO'N and CRL-1472.

To complete gene expression verification, Western blot analysis was performed. All three amplified cell lines (HTB-5, HTB-9 and CRL-1472), but none of the non-amplified cell lines showed strong E2F3 protein expression. HTB-9 exposed highest levels of

E2F3 expression, followed by HTB-5 and CRL-1472. The findings confirm our results from Quantitative real-time PCR analysis. The corresponding protein expression level of NM_017774 was not assessed, because specific antibodies are not available at the moment.

In summary, our comprehensive target gene expression analysis ended up with the conclusion that E2F3 and NM_017774 are without exception co-overexpressed in bladder cancers featuring the 6p22.3 amplification. Clearly, DNA amplification is a perfect method to co-overexpress neighbouring genes. Evidence of clusters of co-overexpressed genes have already been detected in human, fly, and worm **(98)**. Examples in humans include the non-I-integrin alpha-chain genes located in clusters on chromosomes 2, 12, and 17. It has been suggested before that keeping functionally related genes near could be advantageous for a cell because it may ease the burden of unpacking of DNA for transcription **(99)**. It appears possible, that amplification might not always target only one particular gene, but two or more genes that contribute to a common function or pathway. Although only little is known about the possible function of NM_017774, the presence of particular functional domains in the predicted protein structure have linked it to the protein translation machinery **(94)**. It can be expected that a cooperative effect of E2F3 and NM_017774 would result in a particular strong growth advantage and that knock-out of one of these two genes should be sufficient to reverse the effect.

In order to test this hypothesis, we decided to performed knock-out experiments in 6p22.3 amplified cell lines. We chose RNA interference (RNAi) as an established method to specifically inactivate messenger RNA of the selected target genes **(100, 101)**. RNAi is a phenomenon in which the introduction of double-stranded RNA (dsRNA) into a diverse range of organisms and cell types causes degradation of the complementary mRNA. In the cell, long dsRNAs are cleaved into short, 21-25 nucleotide small interfering RNAs, or siRNAs, by a ribonuclease known as Dicer. The siRNAs subsequently assemble with protein components into an RNA-induced silencing complex (RISC). An ATP-generated unwinding of the siRNA activates RISC, which in turn binds to the complementary transcript by base pairing interactions between the siRNA anti-sense strand and the mRNA. The bound mRNA is cleaved and then targeted for destruction. Repeated rounds of sequence specific mRNA degradation finally results in gene silencing **(102, 103)**.

siRNAs are now commonly used as tools to specifically silence target gene expression. Detection and quantification of gene silencing can be assayed by

measuring either mRNA or protein levels, or both. Isolation of high quality RNA is an essential step for subsequent gene expression analysis. But complementary studies at the protein level are also highly recommended, since evidence is mounting that siRNAs can reduce target gene expression at multiple levels: target-specific mRNA degradation, transcriptional regulation through chromatin alteration, and translational control through miRNA-like mechanisms **(104)**. Furthermore, a scrambled control is necessary to assess the specificity of the RNAi-sequence utilized, and to demonstrate the absence of non-specific toxic effects.

Applying RNAi technique resulted in a more than 50% decrease of normal mRNA levels for both candidate target genes. Knock-down of E2F3 strongly inhibited cell proliferation in 6p22.3-amplified cells (-37%), whereas no such effect was observed for NM_017774 (+5%). Combined knock-down of E2F3 and NM_017774 did not result in a stronger proliferation reduction than knock-down of E2F3 alone (-28%). This argues against a cooperative effect of E2F3 and NM_017774 on cell proliferation. Importantly, even after successful knock-down of E2F3 and NM_017774, the residual amount of mRNA left over in the amplified cell line exceeded the standard mRNA levels of non-amplified and non-RNAi treated cells by a factor 5-10. Nevertheless, knock-down of E2F3 severely inhibited regular cell growth in 6p22.3-amplified cells (see **figure 26**). This emphasizes E2F3 as the relevant target gene of 6p22.3-amplification. Together with our recent observation that E2F3 expression is strongly associated with rapid tumour proliferation **(75)**, these data support an important role of E2F3 as a limiting factor for urothelial cell proliferation. It seems that 6p22.3 amplification conveys massive E2F3 overexpression in order to overcome a molecular bottleneck that prevents accelerated cell proliferation.

In contrast, NM_017774 knock-down did not lead to reduced growth rates of 6p22.3 amplified cells. In non-amplified cells, however, reduced levels of NM_017774 induced a comparable proliferation reduction (-49%) as seen for E2F3 (-43%). The growth reduction was even stronger if both E2F3 and NM_017774 were jointly silenced (-58%). The biological function of NM_017774 is unknown yet, but this finding adds additional evidence to the hypothesis (see above) that NM_017774 might be involved in regular cell growth. However, we found no evidence for NM_017774 to have a possible supportive effect when co-amplified and co-overexpressed alongside E2F3.

6p22.3-amplification is strongly linked to rapid tumour proliferation **(75)**. While knock-down of E2F3 severely inhibits regular cell growth of 6p22.3-amplified cells, knock-down of NM_017774 shows no proliferation decrease. Hence, our results suggest that

NM_017774 is only accidentally co-amplified because of its spatial neighbourhood to E2F3 (like other genes in the area), but does not have a functional role in 6p22.3-amplification.

Since our conclusions are largely based on functional analysis we tried to ascertain as carefully as possible that silencing efficiencies were optimal for both E2F3 and NM_017774. Therefore, different RNAi-sequences were tested prior to the functional analysis. Both RNAi-sequences selected for the proliferation assay revealed strong silencing rates, able to reduce target gene expression by at least –50%. Additionally, several different transfection reagents were screened for maximal transfection capabilities versus minimal non-specific toxic effects in combination with selected RNAi-sequences and bladder cancer cell lines. After the optimization period, we obtained excellent transfection rates, surpassing 90%. This is an important achievement since it directly leads to stronger knock-down rates, and thereby more reliable results. Last but not least, all experiments were performed in triplicates, and gene silencing and cell proliferation were carefully observed in parallel at reasonably short intervals and over a considerably long period of time (see **figure 23**). If possible (in case of E2F3), gene silencing was confirmed not only by RT-PCR but additionally be an independent method (W-blot).

It is important to note, that target gene expression was 10 to 20-fold increased in the 6p22.3 amplified cells. As a direct consequence of the high-level amplification, it was impossible to reduce candidate target gene expression to a normal niveau (as prevalent in untreated cell lines without 6p22.3 amplification). Therefore, expression of E2F3 and NM_017774 always remained at least 5 to 10-fold higher in amplified cells, even after experiencing a powerful gene knock-down.

However, silencing of E2F3-expression severely reduced proliferation of 6p22.3 amplified HTB-5 cells. On average the proliferation reduction was –37%, although E2F3 expression rates constantly remained 5 to 10-fold over the limit for regular proliferation. This fact strongly supports the hypothesis, that silencing of E2F3 affects ‘enhanced’ cellular proliferation in 6p22.3-amplified HTB-5. A similar effect can only be suspected for a gene that has the capacity to enhance cellular proliferation in case it is overexpressed. We conclude that E2F3 must be the target gene that drives 6p22.3 amplification in human bladder cancer (**figure 26**).

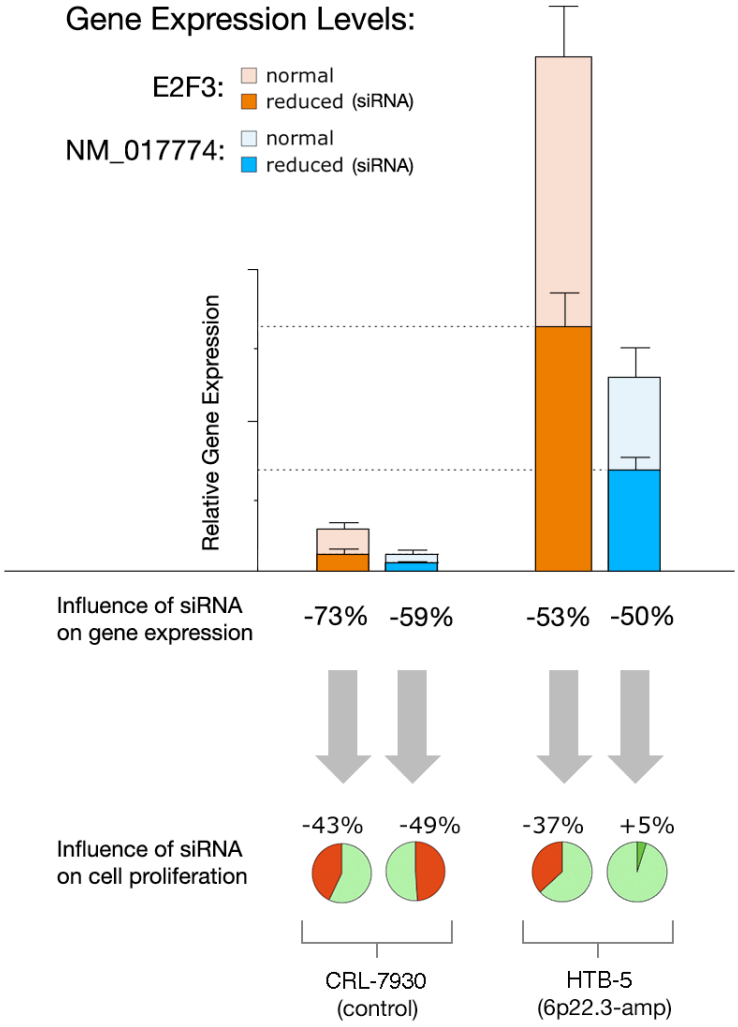

Figure 26: Relative ‚normal' target gene expression and ‚reduced' level after treatment with RNAi. Target gene knock-down surpassed –50% at all times. While knock-down of E2F3 lead to a clear cut proliferation decrease in 6p22.3 amplified HTB-5 cells, knock-down of NM_017774 was unable to show a comparable effect. We therefore conclude that E2F3 must be the responsible gene that drives amplification and/or overexpression of 6p22.3 in bladder cancer.

Based on its biological role E2F3 represents an ideal candidate, whose activation by amplification could easily contribute to oncogenesis. E2F3, which together with E2F1 and E2F2 belongs to the subclass of E2F factors that are thought to act as transcriptional activators of genes that are derepressed following transition through the Rb-dependent G1/S-phase cell cycle checkpoint. Several lines of evidence highlight the importance of E2F3 in cell cycle progression and proliferation. $E2f3^{-/-}$ mouse embryo fibroblasts have a proliferative and cell cycle defect when compared to their wild-type counterparts, and a critical threshold level of one or more E2f3-regulated genes appears to determine the timing of the G1/S transition and rate of DNA synthesis in the mouse **(3, 105, 106)**. Inhibition of E2f3 activity by antibody

microinjection impairs entry into the S-phase, and in transgenic mouse studies E2f3 expression has been demonstrated to contribute to the ectopic proliferation of neuronal cells and lens fibre cells that occur in $Rb^{-/-}$ null mice **(3)**.

It seems that disruption of the Rb-pathway is a particularly important event during bladder cancer development: First, Rb and $p16^{INK4A}$ are the most frequently lost tumour suppressor genes in bladder cancer (along with p53). Second, Rb is the only gene that shows inheritable patterns in bladder cancer. And third, 11q13 (including CCND1) and 6p22.3 (including E2F3) are the most frequently amplified genomic regions in bladder cancer. Overexpressing E2F3 could additionally have the additional advantage for a tumour that it can circumvent the complexity and feedback controls, which exist in upstream signal transduction pathways. Evidence for this theory comes from c-myc, another transcription factor whose expression is tightly regulated in normal cells. In contrast to E2F3, c-myc is only expressed during S-phase of the cell cycle. The chromosomal region harbouring c-myc (8q24) is altered in a large proportion of bladder cancers **(107)**. Overexpression of c-myc is driving tumour cells continuously towards proliferation **(108)**. Alternatively, if the tumour suppressor genes that normally restrict this action by initiating apoptosis are mutated themselves, inappropriate proliferation occurs.

Furthermore, E2F3 may contribute to tumour development and progression in other ways. Recently, it was demonstrated that E2F3 represses p14/ARF, which is an important tumour repressor in the p53 pathway **(39, 40, 55)**. According to these data, overexpression of E2F3 could result in repression of the p53 pathway and thus facilitate tumour progression. It remains to be clarified what kind of selective advantages favour proliferation of tumour cells with amplification of E2F3 and how to measure this effect in an experimental system.

Recent reports of 6p gains in retinoblastoma have detected E2F3 to be amplified and overexpressed also in this tumour type **(109)**. In a similar way as for 6p22.3 amplification in bladder cancer a few years ago, the list of candidate genes for 6p gains in retinoblastoma was narrowed down, but no single gene could be identified as the exclusive target. In addition, it is well known that survivors of hereditary retinoblastoma have a particularly high risk of developing bladder cancer, osteosarcomas, malignant melanomas and small cell lung cancer as secondary tumours **(109)**. It is intriguing to ask whether E2F3 could be involved in these processes, since 6p gains have already been reported for all of these tumour entities **(110-115).** There is a possibility that 6p gains in these tumours may simply target the

same pathway(s) that is /are altered in bladder cancer and vice versa.

When considered with these biological properties of E2F3, our results suggest that the E2F3 gene represents a candidate cancer oncogene that is activated by DNA amplification and/or overexpression. The idea that the E2F3 gene has a role in promoting progression of bladder cancer cells is consistent with the observation that the presence of the 6p22 amplicon in human bladder cancer has been shown to be associated with higher cancer cell proliferation rates **(73)**.

Our data in conjunction with published evidence strongly indicate that amplification of E2F3 is a hallmark of one genetic pathway in invasive bladder cancer that is followed by approximately one third of these tumours.

4.1 Conclusion

Conclusively, the findings of this study document that amplification of 6p22.3 leads to upregulated mRNA expression, and increased protein production of the transcriptionfactor E2F3. While also other genes localized in the amplified region may be co-amplified and co-overexpressed as a by-product of the amplification, E2F3 represents the main target gene and is therefore responsible for the frequent amplification of 6p22.3 in urinary bladder cancer.

4.2 Future Prospects

Mutations in the Rb-pathway are believed to occur in nearly all human cancers **(116)**. Most frequent are mutations in upstream regulators such as the CDK inhibitor $p16^{INK4A}$ and cyclin D1 **(44)**. Mutations in Rb were identified initially in retinoblastoma **(59)** and subsequently in various adult sporadic cancers **(83)**. One of the reasons for studying the functions of E2F has been the idea that it might explain the significance of the Rb-pathway in oncogenesis. Such a role was initially suggested by the identification of E2F as one of the cellular targets for viral oncoproteins **(81, 82)**. Numerous groups have found deregulated E2F activity in different human cancers, often correlating with poor prognosis **(76)**. Many recent discoveries of new family members and new potential target genes have contributed to an increasingly complex view of E2F function reaching beyond G1/S control in several different ways.

Previous analyses of the three bladder cancer cell lines CRL-1472, HTB-5 and HTB-9,

which we have shown to contain amplification and high levels of expression of E2F3, have consistently demonstrated loss or aberrant expression of pRb, and the presence of wild-type INK4A/ARF **(117-120)**. These observations suggest that co-operation between pRb removal and overexpression of E2F3 may be required for bladder cancer carcinogenesis. Two recent publications report 6p22 overexpression (without amplification) in prostate cancer **(79)** and 6p22 gains in retinoblastoma tumours **(109)**. Additional findings of a single case of breast cancer with 6p22.3 amplification in a multitumour TMA experiment, and a second breast cancer that was found to be E2F3 amplified in a DNA-chip based copy number analysis in our lab indicate that E2F3 amplification is perhaps not limited only to bladder cancer. However, true amplification of 6p22.3 has been reported exclusively from the bladder cancer so far **(72, 74, 76)**, but any set of data should be viewed only in the context of a particular environment. Studies of E2F3 function and alterations have to take into account the attributes of the particular biological system such as tissue type, extracellular signals, mutations in other signalling pathways, etc (**figure 27**). It will be interesting to analyse genes upstream and downstream of E2F3 on large multitumour arrays **(121)** to comprehensively study the molecular epidemiology of alterations in the Rb/E2F-pathway of all types of human cancers.

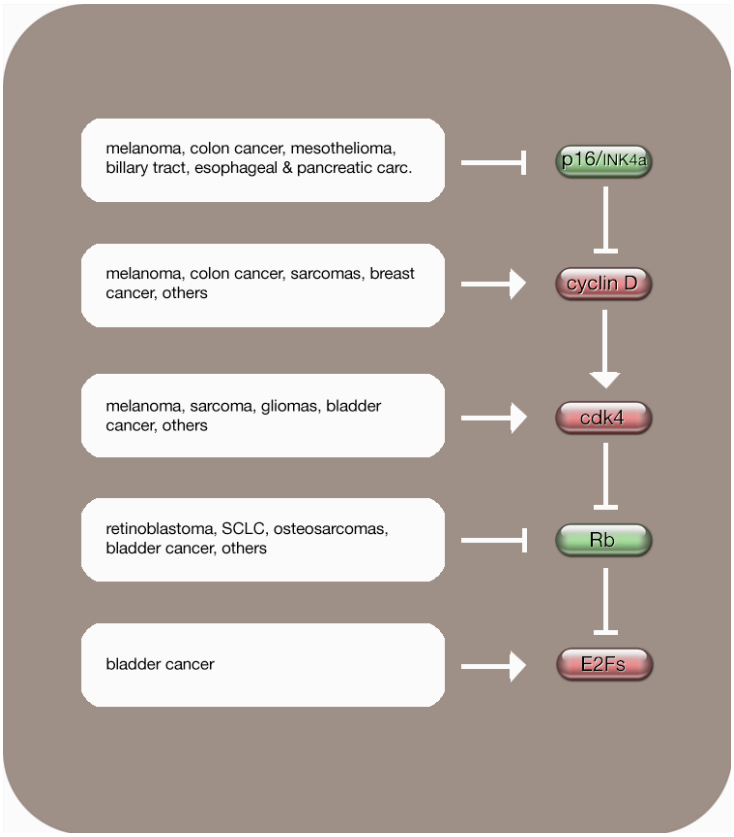

Figure 27: Human tumours often contain alterations such as point mutations, deletions, amplifications or promoter methylations in components of the Rb/E2F pathway. This alterations can be either activating or inactivating. Most frequently, they occur in upstream regulators of Rb. Examples of the types of human cancer where such alterations occur are given next to each component of the pathway. According to *Dimova and Dyson 2005.*

Furthermore, our findings prompt for a review of existing drug compound databases for potential E2F3 inhibitors and may also have relevance to the design of novel drugs targeting cancers with high frequencies of E2F3 amplification and/or overexpression. Drugs directed against upstream targets of E2F3 such as Rb and CDKs **(122)** might be expected to be less effective against cancers overexpressing E2F3. There would also be concern that amplification and upregulation of E2F3 could represent a mechanism for developing resistance to such drugs (**figure 27**).

Last but not least, siRNAs were exploited in this study to resolve the question of the presumptive amplification target gene at 6p22.3. But they are currently also evaluated as potential therapeutic agents **(123)**. If realized, siRNAs could make it possible to target virtually any gene for therapeutic intervention. Synthetic siRNAs and siRNA expression vectors have been injected systemically and into defined tissues and elicited target –specific responses **(124)**. A number of publications have shown that siRNAs can inhibit the replication HIV and Hepatitis B **(125-127)**. As the RNAi field continues to develop, moving into animal models, therapeutics, and drug discovery, eventually reducing E2F3 overexpression in 6p22.3 amplified cancer cells, can perhaps become possible.

5 SUMMARY

The 6p22 is generally regarded as one of the most important amplification sites in urinary bladder cancer. Investigations, encouraged by these findings, subsequently lead to the delineation of the amplicon. During this process the genomic region was narrowed down to 1.7 Mb at 6p22.3, including presumably 13 different genes. Some of these genes were withdrawn from additional investigations due to low-level or absent expression in 6p22.3-amplified bladder cancers. Two genes, however, showed unquestionable correlation between high-level amplification and subsequent overexpression. But the relevant target gene that drives the amplification remained unidentified, yet.

This work was ultimately aimed in a comprehensive comparison of the two remaining candidate oncogenes. The major findings were:

- ✓ By performing FISH on a large bladder cancer TMA we show that NM_017774 is amplified in 11.6% of 893 tested human bladder cancer samples. Thus, the gene reaches an amplification level that is comparable to E2F3.

- ✓ Following case-by-case re-evaluation of a large-section FISH analysis, exhibiting 104 6p22.3-amplified bladder cancers, demonstrates that both genes are 100% co-amplified.

- ✓ Furthermore, we show that both candidate oncogenes are always co-overexpressed in 6p22.3-amplified bladder cancer cell lines, presumably as a consequence of the amplification.

- ✓ Experimentally decreased expression levels of NM_017774 and/or E2F3 similarly lead to strongly inhibited cell proliferation (observed in normal bladder cancer cells CRL-7930; without 6p22.3-amplification).

- ✓ This finding suggests that NM_017774 -the gene of hitherto unknown function –must be functionally connected to the cell cycle regulatory machinery.

- ✓ Besides, decreased E2F3-expression results in proliferation-reduction, and thus confirms the previously predicted essential role of this transcriptionfactor in cell cycle progression.

- ✓ Finally, functional analysis performed in the 6p22.3-amplified HTB-5 cell line, demonstrate that E2F3 –but not NM_017774 -captures a limiting role for enhanced cellular proliferation in 6p22.3-amplified bladder cancer cells.

- ✓ Hence, our results suggest that NM_017774 is only accidentally co-amplified because of its spatial neighbourhood to E2F3 (like other genes in the area), but does not have a functional role in 6p22.3-amplification, whatsoever.

Conclusively, the findings of this study consistently document that amplification of 6p22.3 leads to upregulated mRNA expression, and increased protein production of the transcriptionfactor E2F3. While also other genes localized in the amplified region may be co-amplified and co-overexpressed as a by-product of the amplification, E2F3 represents the main target gene and is therefore responsible for the frequent amplification of 6p22.3 in urinary bladder cancer.

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7 APPENDIX

7.1 Tables

7.2 Figures

7.3 List of Publications

Year	Title	Authors	Journal
2004	E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer.	Oeggerli, M., Tomovska, S., Schraml, P., Calvano-Forte, D., Schafroth, S., Simon, R., Gasser, T., Mihatsch, M. J., and Sauter, G.	Oncogene 2004;23:5616-23
2005	Evaluation of Potential Target Genes of the 6p22.3-Amplicon in Urinary Bladder Cancer.	Oeggerli, M., Schraml, P., Novotny, H., Sauter, G., and Simon, R.	Verh. Dtsch. Ges. Path. 2005;89:000-000
2005	High-throughput tissue microarray analysis of CMYC amplificationin urinary bladder cancer	Zaharieva, B., Simon, R., Ruiz, C., Oeggerli, M., Mihatsch, M. J., Gasser, T., Sauter, G., Toncheva, D.	Int J Cancer; 2005: Dec 20;117(6):952-6
2006	E2F3 is the main target gene of the 6p22-amplicon with high specificity for human bladder cancer	Oeggerli, M., Schraml, P., Bloch, M., Ruiz, Ch., Novotny, H., Mirlacher, M., Sauter, G., and Simon, R.	Oncogene 2006: 25:6538-43

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E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer

Martin Oeggerli[1,3], Sanja Tomovska[1,3], Peter Schraml[1], Daniele Calvano-Forte[1], Salome Schafroth[1], Ronald Simon[1], Thomas Gasser[2], Michael J Mihatsch[1] and Guido Sauter*[,1]

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E2F3 is located in the 6p22 bladder amplicon and encodes a transcription factor important for cell cycle regulation and DNA replication. To further investigate the role of E2F3 in bladder cancer, a tissue microarray containing samples from 2317 bladder tumors was used for gene copy number and expression analysis by means of fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC). E2F3 amplification was strongly associated with invasive tumor phenotype and high tumor grade ($P<0.0001$ each). None of 272 pTaG1/G2 tumors, but 35 of 311 pT1-4 carcinomas (11.3%), had E2F3 amplification. A high E2F3 expression level was associated with high grade, advanced stage, and E2F3 gene amplification ($P<0.0001$ each). To evaluate whether E2F3 expression correlates with tumor proliferation, the Ki67 labeling index (LI) was analysed for each tumor. There was a strong association between a high Ki67 LI and E2F3 expression ($P<0.0001$), which was independent of grade and stage. We conclude that E2F3 is frequently amplified and overexpressed in invasively growing bladder cancer (stage pT1-4). E2F3 expression appears to provide a growth advantage to tumor cells by activating cell proliferation in a subset of bladder tumors.
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Keywords: bladder cancer; 6p22; E2F3; amplification; FISH; IHC

Introduction

Gene amplification plays an important role in the progression of bladder cancer. More than 30 different genomic loci have been identified that recurrently harbor DNA amplifications (Mitelman, 1994; Kallioniemi *et al*., 1995; Voorter *et al*., 1995; Richter *et al*., 1997, 1998; Simon *et al*., 1998; Simon *et al*., 2000). Several of these amplifications contain known oncogenes such as HER2 at 17q21, CCND1 at 11q13, MYC at 8q24, EGFR at 7p13, or MDM2 and CDK4 at 12q13-15 (Dalla-Favera *et al*., 1982; Kondo and Shimizu, 1983; Popescu *et al*., 1989; Xiong *et al*., 1992; Reifenberger *et al*., 1994). The target genes are unknown for the majority of amplicons, such as 1q21-31, 2q13, 3p22-24, 6p22, 8p11, 8q21, 9p21, 10p13-14, 13q13, 13q31-33, 18p11, 20q, 21p11, 22q11-13, Xp11-13, and Xq21-22.2 (Kallioniemi *et al*., 1992; Mitelman, 1994; Sauter *et al*., 1994; Kallioniemi *et al*., 1995; Voorter *et al*., 1995; Richter *et al*., 1997, 1998; Simon *et al*., 1998; Richter *et al*., 1999; Terracciano *et al*., 1999; Zhao *et al*., 1999).

Based on our comparative genomic hybridization (CGH) data of more than 300 bladder carcinomas (Richter *et al*., 1997, 1998; Simon *et al*., 1998; Richter *et al*., 1999; Terracciano *et al*., 1999; Zhao *et al*., 1999; Simon *et al*., 2000), one of the most common sites of high-level amplification is 6p22, which was present in 10 of 172 advanced-stage tumors of our patients. One of the potential candidate genes at the 6p22 region is E2F3 (Veltman *et al*., 2003), which belongs to a family of cell cycle regulatory transcription factors that are controlled by the retinoblastoma tumor suppressor (Lees *et al*., 1993; Leone *et al*., 1998; Humbert *et al*., 2000; Leone *et al*., 2001; Wu *et al*., 2001). Heterodimers of E2F1, E2F2, or E2F3 with DP1 serve as transcriptional activators of genes that promote cell cycling, whereas complexes of E2F/DP with pRb repress transcription and inhibit cell growth (Leone *et al*., 1998; Nevins, 1998; Trimarchi and Lees, 2002). Recent reports have indicated that different members of the E2F gene family could play specific and diverse roles in tumorigenesis of various human malignancies. For example, increased copy numbers and overexpression of E2F1 were found in an erythroleukemia cell line (Saito *et al*., 1995), and E2F5 was amplified and upregulated in 4.2% of breast cancers (Polanowska *et al*., 2000). Decreased expression of E2F1 was to be associated with an increased risk of progression to metastases in bladder cancer (Rabbani *et al*., 1999).

Whereas almost all research on E2F3 has been performed in cell line or mice models so far, little is known about the potential role of E2F3 in human cancers. Based on the rate-limiting role of E2F3 for cell proliferation (Humbert *et al*., 2000), it is possible that a

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disturbed regulation of E2F3 could facilitate cell cycle progression and an increased cell proliferation rate. In turn, overexpression of E2F3 could provide a growth advantage to cells exhibiting this alteration, resulting in clonal selection explaining the presence of 6p22 amplifications in advanced-stage tumors. In this study, we applied the tissue microarray (TMA) technology (Kononen *et al*., 1998) to study E2F3 alterations in urinary bladder cancer. A TMA containing 2317 bladder cancers was used to examine the impact of E2F3 gene copy number changes on the protein expression level, tumor phenotype, and clinical outcome.

Results

Technical aspects

Immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) analyses were performed in a blinded way on different TMA sections. Owing to technical reasons, the number of interpretable samples varies between the individual analyses and comparisons.

E2F3 gene amplification

FISH analyses were performed in two steps: prescreening and establishing optimal hybridization conditions was carried out on a mini-TMA containing specimens with 6p22 amplification (as identified by CGH), followed by a large-scale TMA analysis of 2317 clinical specimens. The 6p22-amplification-specific mini-TMA revealed E2F3 amplification in all three cell lines and seven primary tumors that had shown 6p22 amplification by CGH. The average E2F3 copy numbers in these tumors ranged between 7 and 31.

FISH was successful in 875 of 2317 (38%) samples of the large TMA. FISH-related problems (weak hybridization, background, tissue damage) were responsible for about 80%, TMA-linked problems (too few or absence of tumor cells on the TMA spot) were causing about 20% of the noninformative cases. Statistical analysis was limited to 696 tumors, representing the initial biopsies of 696 patients. E2F3 amplification was detected in 49 these 696 tumors. Examples of amplified and nonamplified tumors are shown in Figure 1. The associations with tumor phenotype are summarized in Table 1. Within urothelial carcinoma, which is by far the most common bladder cancer subtype, E2F3 amplification was strongly associated with high tumor grade and advanced stage ($P<0.0001$ each). Most strikingly, E2F3 amplification was absent in pTaG1/G2 tumors (0 of 272), while 11.3% (35 of 311) of the invasively growing urothelial carcinoma (pT1-4) had E2F3 amplification. E2F3 amplification was most frequent in the histological subgroups of muscle invasive urothelial carcinoma (14.3%) and small cell carcinomas (16.7%).

E2F3 expression in bladder tumor cell lines

To estimate the influence of E2F3 amplification on protein expression, Western blot analysis was performed on three amplified (CRL1479, HTB5, HTB9) and two nonamplified bladder tumor cell lines (CRL7882, RT112). All three amplified, but none of the nonamplified cell lines, showed strong E2F3 protein expression (Figure 2).

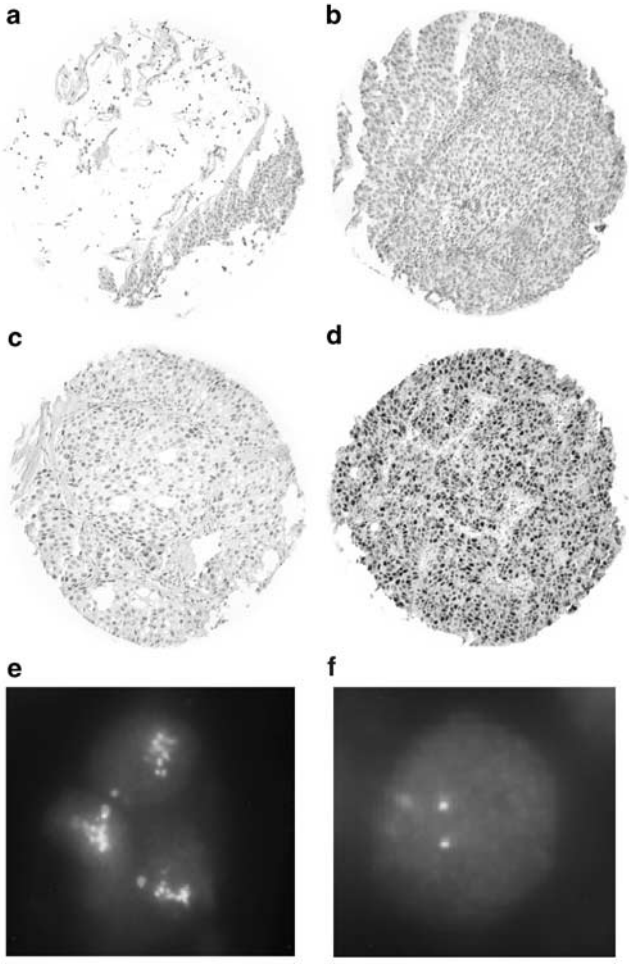

Figure 1 E2F3 alterations in bladder cancer. (**a**) Normal urothelium appeared negative for E2F3 IHC under the selected experimental conditions. (**b–d**) Bladder cancer tissue spots showing different levels of immunostaining. (**d**) Tissue spot of an E2F3-amplified cancer sample showing strong E2F3 expression. All tissue spots (**a–d**) are located on the same TMA slide and have been subjected to identical experimental conditions. (**e**) E2F3 gene amplification as detected by FISH analysis. Red fluorescence signals represent centromere 6 copies, massive increase of green E2F3 signals indicate gene amplification. (**f**) Tumor cell nuclei with two E2F3 and centromere 6 copy numbers, representing the normal copy number state

E2F3 IHC on tumor microarrays

E2F3 IHC was analysed within 1334 first biopsy tumor tissue spots on the bladder cancer TMA. Nearly one-fifth of the tumors exhibited varying degrees of positive staining. No E2F3 staining was observed in normal urothelium (Figure 1a). Examples of E2F3-positive and -negative tumors are shown in Figure 1b–d. E2F3 expression by IHC was significantly linked to E2F3 copy

Table 1 E2F3 alterations and tumor phenotype in urinary bladder cancer

	E2F3 FISH			*E2F3 IHC*		
	n[a]	*Amplified (%)*	P[b]	n[a]	*Positive (%)*	P[b]
All tumors	696	7.0		1334	17.3	
Histology[c]						
Urothelial carcinoma	140	14.3		337	21.1	
Squamous cell carcinoma	14	7.1		38	18.4	
Small cell carcinoma	12	16.7		18	55.6	
Sarcomatoid carcinoma	6	0.0		10	0.0	
Adenosquamous carcinoma	1	0.0		1	0.0	
Stage[d]						
pTa	301	0.3		539	11.1	
pT1	171	8.8	*P<0.0001*[e]	271	20.7	*P<0.0001*[e]
pT1-	27	18.5		46	26.1	
pT2–4	140	14.3		337	21.1	
Grade[d]						
G1	73	0.0		163	8.0	
G2	315	1.3	*P<0.0001*	527	10.8	*P<0.0001*
G3	254	14.6		507	26.0	
Stage and grade[d]						
pTa G1	73	0.0		162	8.0	
pTa G2	199	0.0		330	10.9	
pTa G3	29	3.4		47	23.4	
pT1 G2	70	1.4		107	9.3	*P<0.0001*
pT1 G3	101	13.9	*P<0.0001*	164	28.0	
pT2–4 G2	35	5.7		79	8.9	
pT2–4 G3	105	17.1		258	24.8	
Growth pattern[c,d]						
Papillary	58	13.8	*0.4292*	126	20.6	*0.6486*
Solid	81	14.8		210	21.4	

[a]Number of tumors with interpretable results (only first biopsies are considered). [b]Chi-square test. [c]Only pT2-4. [d]Only urothelial ca. [e]pT1-excluded

number changes ($P<0.0001$). E2F3 positivity was found in 24 of 34 amplified urothelial carcinomas (70.6%), but in only 82 of 456 (18.0%) nonamplified tumors. This association held also true within the group of 269 pT1-4 urothelial carcinomas that could be analysed by both FISH and IHC ($P<0.0001$). E2F3 positivity was significantly more frequent in small cell carcinomas (55.6% positive) than in other histologic subtypes (Table 1). Within urothelial carcinomas, E2F3 expression was linked to advanced stage and high grade ($P<0.0001$ each). The frequency of E2F3-positive tumors increased from 10% in pTaG1/G2 tumors (49 of 492) to 20.9% in invasively growing pT1-4 urothelial carcinomas (127 of 608).

E2F3 expression and tumor cell proliferation (Ki67 labeling index (LI))

Both gene amplification and protein overexpression were significantly associated with rapid tumor cell proliferation ($P<0.0001$ each). Analysis of variance (ANOVA) analysis including E2F3 expression/amplification and either tumor stage or grade showed that both E2F3 expression and amplification were independent predictors of rapid tumor cell proliferation ($P<0.0001$ each). Accordingly, the separate analyses of tumors of identical grades and stages lead to either significant differences in the proliferation between E2F3-negative and -positive tumors or at least to a clear tendency towards a higher Ki67 LI in E2F3-positive tumors (Table 2).

E2F3 alterations and prognosis

E2F3 expression was associated with poor tumor-specific survival if all patients were included in the analysis ($P<0.05$,. Figure 3). There was no association between E2F3 expression and tumor-specific survival within the subgroup of pT2-4 urothelial carcinomas, or between E2F3 alterations and tumor recurrence or tumor progression among pTa/pT1 tumors. There were too few E2F3-amplified tumors with available survival data to allow statistically meaningful analysis.

Discussion

Previous CGH studies have repeatedly highlighted 6p22 as an amplification site in bladder cancer (Richter *et al*., 1998, 1999). E2F3, a key gene for G1/S transition (Leone *et al*., 1998), has been mapped to 6p22. Studies by array CGH have suggested that E2F3 can be

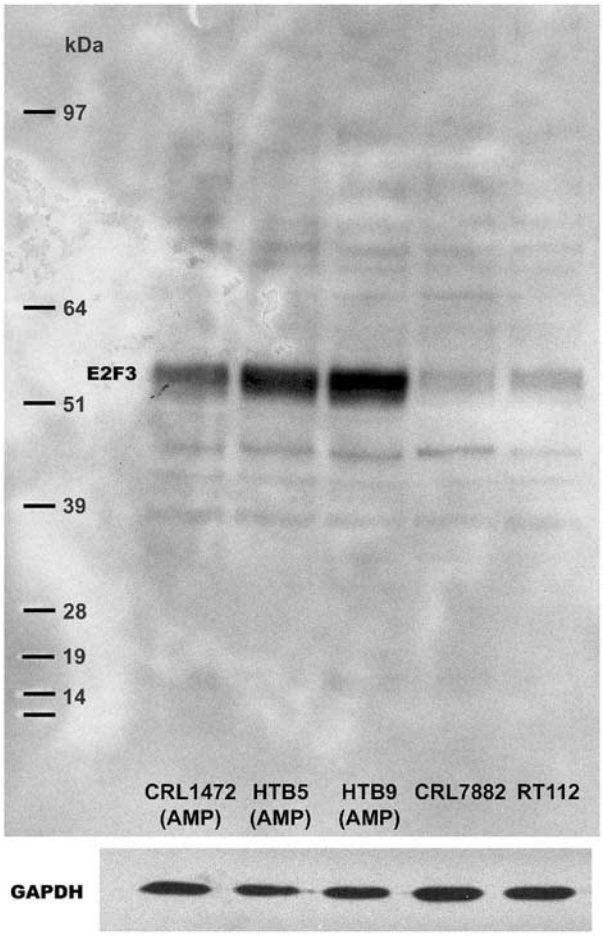

Figure 2 Western blot analysis of E2F3 in 6p22-amplified bladder cancer cell lines (CRL1472, HTB5, HTB9) and in nonamplified cell lines (CRL7882, RT112). Amplified cell lines show a massive increase of E2F3 protein expression as compared to nonamplified bladder cancer cell lines. Weak nonspecific bands are seen in both amplified and nonamplified cell lines

included in the 6p22 amplicon in at least a fraction of bladder tumors (Veltman *et al*., 2003). In an attempt to further investigate the importance of E2F3 in primary bladder tumors and in bladder cancer cell lines, we first analysed a mini-TMA composed of 10 tumors with known 6p22 amplification (by CGH). Our finding of an involvement of E2F3 in the 6p22 amplicons of all 10 amplified tumors provided strong evidence for a major role of E2F3 in bladder cancer. Western blot analysis targeting the E2F3 protein showed strong E2F3 overexpression in all amplified cell lines demonstrating a functional relevance of E2F3 amplification. Based on these data, we proceeded to analyse the prevalence and significance of E2F3 amplification/expression in our previously constructed bladder cancer TMA-containing tissues from 2317 different tumors (Richter *et al*., 2000).

The TMA approach allowed the rapid analysis of more than 800 carcinomas on the DNA and protein level. The findings suggest an important role of E2F3 amplification/overexpression in invasive bladder cancer. A total of 14.3% of muscle invasive bladder cancers showed E2F3 amplification. This makes E2F3 one of the most frequently amplified genes in invasive bladder cancer. Using the same methodological criteria, including FISH protocols, copy number cutoff levels, and TMA resources, we have observed similar frequencies for amplifications of HER2 (14%) (Simon *et al*., 2003) and CCND1 (15%) (Zaharieva *et al*., 2003) in pT2-4 bladder cancer. Remarkably, E2F3 amplification was not found in any of 272 pTaG1/G2 tumors, which is consistent with models suggesting that pTaG1/G2 tumors are genetically stable neoplasias with a much lower likelihood to acquire chromosomal rearrangements than invasively growing tumors (Richter *et al*., 1997, 1998; Simon *et al*., 1998; Richter *et al*., 1999; Zhao *et al*., 1999; Simon *et al*., 2001, 2002). Recently, chromosomal alterations have been successfully used for the detection of bladder cancer in voided urine cells (Halling *et al*., 2000; Bubendorf *et al*., 2001) and a commercial FISH test has been approved by the US Food and Drug Administration in 2001. Our data raise the possibility that 6p22 amplification detection could have clinical utility for distinction of invasive and noninvasive bladder tumors in urine cells.

Given the well-known function of E2F3 for S-phase induction (Leone *et al*., 1998), it could be expected that E2F3 overexpression exerts an oncogenic function through cell cycle activation. This hypothesis is largely supported by the strong association of E2F3 amplification and expression with tumor cell proliferation (Ki67 LI), which was also found in all large subgroups of tumors with identical grade and stage. Despite this strong link between E2F3 positivity and a high Ki67 LI, no association was found between E2F3 overexpression and prognosis if tumors with comparable tumor stages were examined. This result is not completely unexpected. Although there are studies suggesting a prognostic role of tumor cell proliferation in urinary bladder cancer (Lipponen *et al*., 1993; Liukkonen *et al*., 1999), there are also reports that fail to reproduce these data (Nakopoulou *et al*., 1998; Pfister *et al*., 1998). Our own previous analyses on the same set of carefully staged tumors were unable to show a convincing association of Ki67 LI with prognosis in pTa, pT1, or pT2-4 cancers (Nocito *et al*., 2001). Although accelerated cell proliferation is an important prerequisite for tumor growth, it is probably other factors, like invasive growth and the metastatic potential, which are the key determinants for the clinical outcome of neoplastic diseases.

The comparison of E2F3 FISH and IHC data revealed a good but not a perfect correlation. A strong E2F3 expression by IHC was seen in 70% of E2F3-amplified, but in only 18% of nonamplified tumors. These data seem to suggest that E2F3 overexpression is not present in all amplified tumor samples. However, at least some of the discrepant results might be caused by technical reasons. For example, none of the currently commercially available antibodies targeting E2F3 (including that one used in our study) is specifically recommended for IHC on formalin-fixed tissues. It is possible that technical shortcomings of our IHC procedure resulted in a fraction of IHC false-negative

Table 2 E2F3 amplification/overexpression in relation to Ki-67 LI

	E2F3 FISH			*E2F3 IHC*		
	Nonamplified	*Amplified*	P[a]	*Negative*	*Positive*	P[a]
pTaG1	73[b] 5.7[c] (4.5–6.6)[d]	0		147 6.3 (5.4–7.2)	13 7.4 (4.4–10.3)	0.4929
pTaG2	199 10.1 (9.0–11.2)	0		294 10.2 (9.2–11.1)	36 13.8 (11–16.6)	0.015
pTaG3	28 22.0 (17.2–26.8)	1 35.3 (9.9–60.6)	0.2984	36 22.8 (18.4–27.2)	11 24.2 (16.3–32.1)	0.7512
pT1G2	69 19.1 (16.2–22.0)	1 14.9 (−9.2 to 38.8)	0.7252	96 19.1 (16.7–21.5)	10 24.7 (17.3–32.1)	0.1567
pT1G3	86 28.9 (25.6–32.2)	14 45.9 (37.7–54.1)	0.0002	118 28.8 (26–31.6)	46 37.6 (33.1–42.1)	0.0012
pT2G2	33 21.4 (17.0–25.8)	2 36.5 (18.7–54.2)	0.1019	72 21.9 (18.9–24.8)	7 35.9 (26.5–45.4)	0.0059
pT2G3	87 35.0 (31.4–38.5)	18 44.4 (36.6–52.2)	0.0305	192 31.4 (29.1–33.7)	63 44.1 (40.2–48.1)	<0.0001

Only first biopsies of patients with TCC ($n = 1853$) included. Nonamplified cases include gains. [a]Student's *t*-test. [b]Mean Ki-67 LI. [c]95% CI of Ki-67 LI. [d]Number of samples

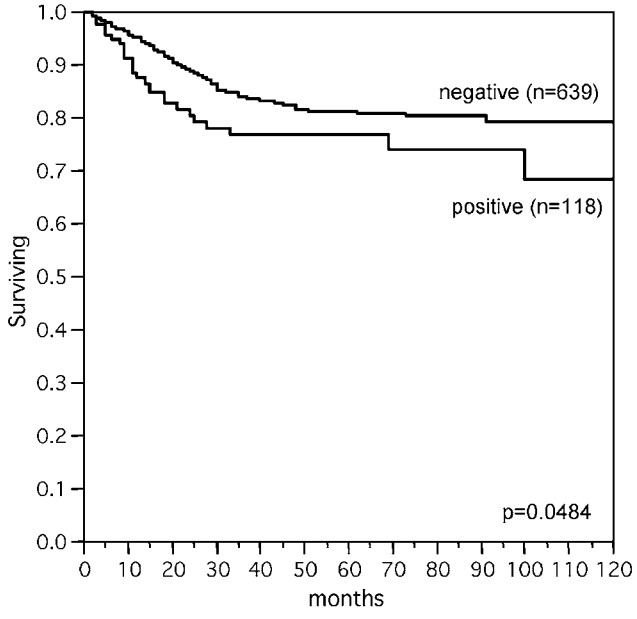

Figure 3 Survival analysis (Kaplan–Meier plot) in a subset of 757 urothelial carcinoma samples with data on E2F3 IHC and patient outcome. Immunohistochemically positive cancers show shortened tumor-specific survival

samples. Alternatively, our data could indicate that E2F3 is not the (only) amplification target at 6p22. Overexpression of one or several other genes in the same amplicon could be required to drive the growth advantage of amplified cells in 6p22-amplified tumors without E2F3 protein overexpression. There is a growing evidence indicating that the molecular mechanism of gene amplification does not follow the simple one gene–one amplicon concept. Amplification may be a mechanism that is particularly effective to simultaneously overexpress multiple adjacent genes, which may jointly provide a growth advantage to amplified tumor cells. For example, neighboring oncogenes that sometimes undergo coamplification have recently been identified at various genomic regions such as MDM2, GLI, CDK4, and SAS at 12q13-q15 (Reifenberger *et al*., 1994) or CCND1, EMS1, and INT2 at 11q13 (Hui *et al*., 1997).

Only few known genes are known to be located in direct genomic neighborhood of E2F3. A gene of unknown function (GenBank: NM_017774) maps closely centromeric to E2F3. The 579 amino-acid protein contains a domain that is present in several other proteins associated with the translation machinery and in a family of small, uncharacterized archaeal proteins that are predicted to play a role in the regulation of tRNA modification or translation (Anantharaman *et al*., 2001). SOX4, a transcription factor that is a member of the high mobility group (HMG)-box family of DNA binding protein (Farr *et al*., 1993), and PRL (the gene encoding Prolactin) map to region between 1 and 2 Mb centromeric to E2F3. Prolactin (PRL) is a protein hormone closely related to growth hormone and mainly secreted in the anterior pituitary lactotrope and the

decidualized stromal cell of the human endometrium (DiMattia *et al*., 1990). However, Northern analysis demonstrated no correlation between amplification and overexpression of these genes in eight cell lines (Bruch *et al*., 2000).

In summary, E2F3 is regularly included in a bladder cancer amplicon at 6p22. Taking together the known cell cycle activating role of E2F3, its overexpression in amplified tumors, and the association with cell proliferation *in vivo*, it appears that E2F3 could be an important target gene inside the 6p22 amplification whose overexpression gives growth advantage to amplified tumor cells. This study also demonstrates how a combination of genomic technologies, microarray discovery platforms, and bioinformatics resources can be used to rapidly identify, validate, and characterize target genes for genetic alterations and associate these changes to specific medical conditions.

Materials and methods

CGH

A review of the CGH profiles of 278 primary bladder carcinomas and 20 cell lines previously examined in our laboratory revealed 10 tumors and three cell lines with distinct peaks around 6p22. Examples of CGH profiles are shown in Figure 4.

Bladder cancer tissue microarray (TMA)

The composition of our bladder cancer TMA containing 2317 formalin-fixed paraffin-embedded tissues from 1853 bladder cancer patients was previously described (Richter *et al*., 2000; Simon *et al*., 2002). Some of the clinical data were updated for this study. All slides of all tumors were reviewed by one pathologist (GS). Tumor stage and grade were defined according to UICC and WHO (Mostofi, 1973; UICC, 1992). Stage pT1 was defined by presence of both unequivocal tumor invasion of the suburothelial stroma and tumor-free fragments of the muscular bladder wall. Carcinomas with stroma invasion but absence of muscular bladder wall in the biopsy were classified as at least pT1 (pT1-). Clinical data were available from 1123 patients. The medium follow-up period was 42 months (range 1–236 months). Time to recurrence and time to progression (to stage pT2 or higher) were selected as study end points for patients with pTa and pT1 tumors.

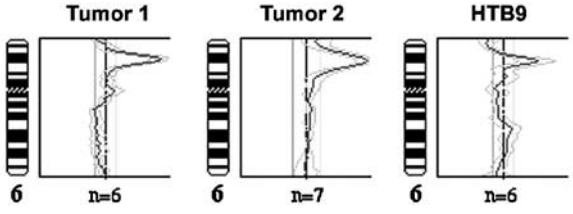

Figure 4 Examples of 6p22 amplifications in bladder cancer as detected by CGH. The central line indicates the fluorescence ration of balanced DNA sequence copy number state (1.0), lines to the left and right represent the 0.8 and 1.2 thresholds for losses and gains. 6p22 amplifications are indicated by strong shifts of the fluorescence ratio profile to the right in the respective chromosomal regions

Follow-up information was considered complete enough to include a pTa/pT1 cancer patient in the study if cystoscopies had been performed at least at 3, 9, and 15 months, then annually until the end point of this study (recurrence, last control). To include a patient for analyses of time to progression, longer intervals between controls were accepted if the last follow-up control ruled out progression. Recurrences were defined as cystoscopically visible tumors. Tumor progression was defined as the presence of muscle invasion (stage pT2 or higher) in a subsequent biopsy. An overview of the histological and clinical data is given in Table 3.

FISH

The tissue microarray sections were treated according to the Paraffin Pretreatment Reagent Kit protocol (purchased from Vysis, Downers Grove, IL, USA) before hybridization. FISH was performed with a digoxigenated PAC probe (PAC

Table 3 Histological and clinical parameters of 2317 arrayed bladder cancer samples

	Tumors (n = 2317)	*Patients (n = 1853)*	*Patients with clinical data (n = 1123)*
Stage			
pTa	951	768	502
pT1	515	425	263
pT1-	101	80	34
pT2–4	737	571	320
Grade			
G1	282	230	157
G2	987	792	467
G3	1048	831	498
Stage/grade			
pTaG1	277	226	155
pTaG2	567	461	291
pTaG3	107	81	56
pT1G2	206	170	98
pT1G3	309	255	165
pT2–4G2	186	140	70
pT2–4G3	551	431	250
Histology			
Transitional cell carcinoma	2108	1678	1032
Squamous cell carcinoma	73	59	34
Small cell carcinoma	31	25	12
Adenocarcinoma	22	17	8
Adenosquamous carcinoma	2	2	1
Sarcomatoid carcinoma	24	17	8
Growth pattern			
Papillary	1665	1367	868
Solid	633	472	249
No. of tumors per patient			
One	1533	1533	914
Two or more	784	320	209
Clinical end points	—	—	
Tumor-specific survival (pT2-4)	—	—	320
Time to progression (pTa and pT1)	—	—	482
Time to recurrence (pTa and pT1)	—	—	535

dJ177P22, Sanger Centre, UK) containing the E2F3 gene and a Spectrum Red-labeled chromosome 6 centromeric probe (CEP6) as a reference (purchased from Vysis). Hybridization and posthybridization washes were according to the 'LSI procedure' (Vysis). Probe visualization using fluorescent isothiocyanate (FITC)-conjugated sheep anti-digoxigenin (Roche Diagnostics, Rotkreuz, Switzerland) was as described (Wagner *et al*., 1997). Slides were counterstained with 125 ng/ml 4′,6-diamino-2-phenylindole in an antifade solution. Amplification was defined as presence (in $\geqslant 5\%$ of tumor cells) of at least three times as many E2F3 gene signals than centromere 6 signals.

IHC

Standard indirect immunoperoxidase procedures were used for IHC (ABC-Elite, Vector Laboratories, Burlingame, CA, USA). The monoclonal antibody E2F3 Ab-4 (Lab Vision Corporation, CA, USA) was tested on array sections containing formalin-fixed paraffin-embedded, E2F3-amplified and nonamplified bladder tumors. Optimal staining of the cell nuclei (1 : 100 dilution of primary antibody) could best be achieved after pretreatment in 1 mM EDTA at 99°C for 40 min for antigen retrieval. The primary antibody was omitted for negative controls. Diaminobenzidine was used as a chromogen. Some cytoplasmic staining was seen in most tissue spots but only nuclear staining was scored. The IHC staining intensity (scored in a four step scale including 0, 1+, 2+, and 3+) and the fraction of positive tumor cells was recorded for each tissue spot. Based on these values, a final IHC result was calculated according to the following criteria: Negative: no staining at all, or 1+ staining intensity in no more than 10% of tumor cells; positive: at least 2+ staining intensity in more than 10% of tumor cells.

The rabbit monoclonal antibody MIB1 (1 : 800, Dianova, Hamburg, Germany) was employed to detect Ki67 protein (expressed in all cells in G1, S, G2, and M phase) as previously described (Moch *et al*., 1997). Nuclei were considered Ki67 positive if any nuclear staining was seen. The Ki67 LI (percentage of Ki67-positive cells) was determined on each arrayed tumor sample by scoring at least 300 cells each. Tumors with Ki67-negative mitoses were excluded from analyses.

Western analysis

Protein was extracted from about 2×10^6 cells from bladder cancer cell lines CRL1472, HTB5, HTB9, CRL7882, and the RT112 cell line as described (Leone *et al*., 1998). In all, 10 μg protein of each sample was subjected to SDS–PAGE on 10% polyacrylamide gels. Proteins were transferred onto PVDF membrane (Bio-Rad, Glattbrugg, Switzerland). The membrane was blocked in TBS (25 mM Tris at pH 7.4, 137 mM NaCl, 2.7 mM KCl) containing 10% skim milk at RT for 2 h. Blots were then incubated with mouse monoclonal E2F3 Ab-4 antibody (5 μg/ml) (Lab Vision, CA, USA), which is directed against the E2F3 full-length protein, in TBS containing 5% skim milk overnight at 4°C, and washed subsequently in TBS containing 0.1% Tween 20 for 30 min. Blots were incubated with a second antibody (1 : 2000) (goat anti-mouse IgG, Fc, AP127P; Juro Supply AG, Lucerne, Switzerland) for 1 h at RT, and washed for 30 min. Blots were processed with the ECL system (Amersham Pharmacia Biotech, Dubendorf, Switzerland).

Statistics

All tissue samples on the TMA were utilized for comparisons of amplification and overexpression of E2F3. Only the first biopsy was used for further statistical analyses in patients having more than one tumor on the TMA. Contingency table analysis and Chi-square tests were applied to study the relationship between histology tumor type, grade, stage, and E2F3 expression/amplification. Student's *t*-tests were employed to examine the associations of the Ki67 LI with E2F3 expression/amplification. ANOVA was utilized to determine the parameters with greatest influence on tumor cell proliferation. Survival curves were plotted according to the Kaplan–Meier method and analysed for statistical differences using a log rank test. Patients with pTa/pT1 tumors were censored at the time of their last clinical control showing no evidence of disease or at the date when cystectomy was performed. Patients with pT2-4 carcinomas were censored at the time of their last clinical control or at the time of death if they died from causes not related to their tumor.

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High-throughput tissue microarray analysis of *CMYC* amplification in urinary bladder cancer

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Alterations of chromosome 8, preferentially deletions of 8p and gains of 8q, belong to the most frequent cytogenetic changes in bladder cancer. *CMYC* on 8q24 is a candidate oncogene in this region. Little is known about the clinical significance of *CMYC* copy number changes in urinary bladder cancer because its frequency is low and a limited numbers of tumors were analyzed so far. To investigate the impact of *CMYC* alterations on tumor progression and patient prognosis in bladder cancer, we applied FISH to a tissue microarray containing 2317 bladder cancer samples. Presence of *CMYC* copy number increase was associated with advanced stage and high grade. *CMYC* amplifications were seen in 3 of 467 pTa (0.6%), 10 of 247 pT1 (4%) and 11 of 201 pT2–4 urothelial carcinomas (5.5%; $p < 0.0001$), as well as in 1 of 123 G1 (0.8%), 8 of 470 G2 (1.7%) and 17 of 365 G3 urothelial carcinomas (4.7%; $p < 0.0001$). *CMYC* gains were present in 49 of 467 pTa (10.5%), 39 of 247 pT1 (15.8%) and 43 of 201 pT2–4 urothelial carcinoma (21.4%; $p < 0.0001$), as well as in 7 of 123 G1 (5.7%), 56 of 470 G2 (11.9%) and 72 of 365 G3 urothelial carcinomas (19.7%; $p < 0.0001$). *CMYC* copy number changes were unrelated to prognosis of bladder cancer patients. We conclude that alterations of the *CMYC* gene, including copy number gains and amplifications, are linked to genetically unstable bladder cancers that are characterized by a high histologic grade and/or invasive growth. Patient prognosis was not affected by *CMYC* gene copy number changes.

Key words: bladder cancer; *CMYC*; fluorescence *in situ* hybridization; tissue microarray

Amplification of *CMYC* was discovered in a number of human cancers, including breast, lung, renal, prostate, head and neck, endometrium and colorectal carcinomas.[1–8] *CMYC* encodes a transcription factor containing DNA-binding sequences. It permits entry into the cell cycle by activating several genes, *e.g.*, those encoding ornithine decarboxylase and the protein phosphatase cdc25[9,10] and by repressing others, *e.g.*, encoding tissue-specific transcription factors.[11,12]

Alterations of *CMYC* gene copy numbers or expression levels are known to occur in bladder cancer. Some authors reported association of CMYC overexpression with histologic grade,[13,14] while others rejected such a relation.[15–18] With regard to tumor progression, previous studies either did not find an association[15–19] or linked CMYC expression to low-grade early-stage tumors.[20,21] Increased CMYC protein levels may occur as a consequence of gene copy number changes, such as gains of the whole long arm of chromosome 8 or *CMYC*-specific high-level gene amplification, but have been also been reported in tumors without evident changes in the *CMYC* gene copy number.[17,20,22–24]

Increased *CMYC* gene copy numbers were shown to be significantly associated with late-stage and high-grade tumors.[20,24] A recent study suggested that increased *CMYC* copy numbers might predict invasive tumor growth.[25] Little is known about the prognostic value of *CMYC* alterations in bladder cancer, probably because of the rarity of *CMYC* amplifications occurring in less than 10% of cases.[26–29] High numbers of tumors must therefore be analyzed to allow for statistically relevant comparisons. Tissue microarrays (TMAs) are optimally suited to determine associations between tumor phenotype and genotype or prognosis.[30] Here, we utilized our previously described bladder cancer prognosis tissue microarray (TMA)[28,29,31] to establish the prognostic role of *CMYC* amplifications in urinary bladder cancer.

Material and methods

Bladder cancer tissue microarray

A preexisting bladder cancer TMA containing 2,317 samples from 1,853 patients was used.[31] The slides of all tumors were reviewed prior to construction by a single pathologist (G.S.). Tumor stage and grade were defined according to Union Internationale contre le Cancer and World Health Organization criteria.[32,33] Because of the limitations of transurethral biopsies in accurately determining the depth of invasion of higher-stage bladder cancer, all tumors showing muscle invasion were categorized in one group (pT2–4). Tumors confined to the bladder mucosa were classified as stage pTa. Stage pT1 was defined by the presence of both unequivocal tumor invasion of the suburothelial stroma and tumor-free fragments of the muscular bladder wall. Carcinomas with stroma invasion but absence of muscular bladder wall in the biopsy were classified as at least pT1 (pT1–). A papillary tumor growth was assumed if at least one unequivocal papilla with similar atypia as in the invasive tumor area was present. An overview of the histologic and clinical data is given in Table I. The numbers in the subgroups do not always add up to the total number of samples because of missing data. We did not reduce the data set to samples where all data are available because it would lead to unnecessary loss of statistical power in the subgroup analyses. No data had been recorded for histologic tumor type ($n = 57$), stage ($n = 13$) and tumor growth pattern ($n = 19$) in small subsets of tumors. Of the 1,853 patients, 1,384 were males and 465 females. The gender was unknown in 4 patients. The average age was 68 years (range, 20–100 years). Clinical data were available from 1,123 patients. The medium follow-up period was 42 months (range, 1–272 months). Time to recurrence and time to progression (to stage pT2 or higher) were selected as study endpoints for patients with pTa and pT1 tumors. Follow-up information was considered complete enough to include a pTa/pT1 cancer patient in the study if cystoscopies had been performed at least at 3, 9 and 15 months, then annually until the endpoint of this study (recurrence, last control). To include a patient for analyses of time to progression, longer intervals between controls were accepted if the last follow-up control ruled out progression. Recurrences were defined as cystoscopically visible tumors. Tumor pro-

Abbreviations: CEP, centromere enumeration probe; CGH, comparative genomic hybridization; DAPI, 4,6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; TCC, transitional cell carcinoma; TMA, tissue microarray.

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TABLE I – HISTOLOGIC AND CLINICAL PARAMETERS OF 2,317 ARRAYED BLADDER CANCER SAMPLES

	Tumors (n = 2,317)	Patients (n = 1,853)	Patients with clinical data (n = 1,123)
Stage			
pTa	951	768	502
pT1	515	425	263
pT1–	101	80	34
pT2–4	737	571	320
Grade			
G1	282	230	157
G2	987	792	467
G3	1,048	831	498
Stage/grade			
pTaG1	277	226	155
pTaG2	567	461	291
pTaG3	107	81	56
pT1G2	206	170	98
pT1G3	309	255	165
pT2–4G2	186	140	70
pT2–4G3	551	431	250
Histology			
Transitional cell carcinoma	2,108	1,678	1,032
Squamous cell carcinoma	73	59	34
Small cell carcinoma	31	25	12
Adenocarcinoma	22	17	8
Adenosquamous carcinoma	2	2	1
Sarcomatoid carcinoma	24	17	8
Growth pattern			
Papillary	1,665	1,367	868
Solid	633	472	249
Number of tumors per patient			
One	1,533	1,533	914
Two or more	784	320	209
Clinical endpoints			
Tumor-specific survival (pT2–4)			320
Time to progression (pTa and pT1)			482
Time to recurrence (pTa and pT1)			535

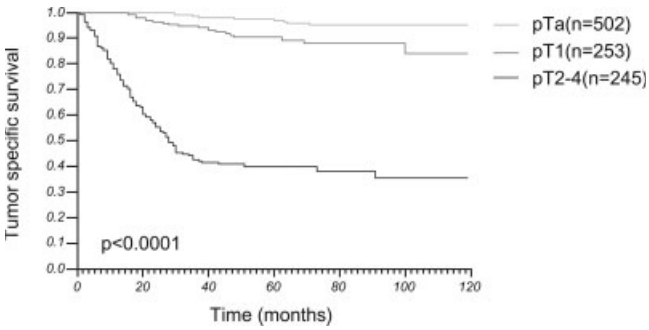

FIGURE 1 – Influence of tumor stage on tumor-specific survival in 1,000 bladder cancer patients (numbers do not add up to the total number of patients analyzed in this study since patients with nonurothelial cancers or unclear stage diagnosis were excluded).

gression was defined as the presence of muscle invasion (stage pT2 or higher) in a subsequent biopsy. Treatment conditions (TUR/cystectomy) were known for 500 pTa (TUR), 230 pT1 (221 TUR, 9 cystectomy) and 175 pT2–4 (cystectomy) cancers. For survival analysis of pT2–4 tumors, it was assumed that at least the majority of the 195 patients with unknown treatment underwent cystectomy. The expected differences in survival between our pT1 and pT2–4 patients (Fig. 1) strongly support the validity of this assumption.

Fluorescence in situ *hybridization (FISH)*

Prior to hybridization, the slides were treated according to the Paraffin Pretreatment Reagent Kit (Vysis, Downers Grove, IL). FISH was performed using combined CEP8 Spectrum Green/*CMYC* Spectrum Orange-labeled probe (Vysis). Denaturation of the DNA was carried out at 75°C for 10 min (probe mixture) or 5 min (slides). The probe mixture was applied to the slides and hybridized overnight in a moist chamber at 37°C. The posthybridization washes were performed as described in ''LSI procedure'' (Vysis). Slides were counterstained with 125 ng/ml DAPI in antifade solution. The counts for *CMYC* and CEP8 were estimated in all tissue spots. Two different approaches were made to define *CMYC* copy number gains. In a first analysis, tumors showing an average of 3 or more gene signals (averaged per tissue spot) were recorded as increased gene number. In the second approach, the presence of more than 2 times more gene signals than corresponding centromere signals of chromosome 8 in at least 50% of tumor cells was considered as amplification, while the presence of more gene signals than centromere 8 signals (but not reaching the definition for amplification) in at least 50% of tumor cells was considered as a gain. All other tumors were considered normal for the purpose of this study.

Statistics

Only the first biopsy of patients having more than one biopsy on the array was used for statistical analyses. Contingency table analysis and chi-square tests were used to study the relationship between histologic tumor type, grade, stage and gene amplification. Analysis of variance (ANOVA) test was applied to evaluate the influence of *CMYC* copy numbers on tumor phenotype. Survival curves were plotted according to Kaplan-Maier.[34] A log-rank test was applied to estimate the relationship between grade, stage, or gene amplifications and tumor recurrence, progression or survival. Patient with pTa/pT1 tumors were censored at the time of their last clinical control showing no evidence of disease or at the date when cystectomy was performed. For survival analysis, patients were censored at the time of their last clinical control or at the time of death if they died from causes not related to their tumor.

TABLE II – *CMYC* COPY NUMBERS, GAIN, AMPLIFICATION STATUS AND TUMOR PHENOTYPE

	n[1]	Absolute normal (%)	*CMYC* copy number increased (%)	p[4]	Mean value[5]	Relative *CMYC* copy number			
						Normal (%)	Gain (%)	Amplification (%)	p[4]
Histology[2]	1,052	79.2	20.8		2.5	83	14.1	2.9	
Transitional cell carcinoma	20	64.2	35.8		3	73.1	21.4	5.5	
Squamous cell carcinoma	29	82.8	17.2	0.0373[7]	2.3	79.3	17.2	3.4	0.7515[7]
Small cell carcinoma	12	58.3	41.7	0.6849[7]	4	91.7	0	8.3	0.0602[7]
Sarcomatoid carcinoma	11	72.7	27.3	0.5561[7]	2.3	72.7	18.2	9.1	0.8786[7]
Adenocarcinoma	5	80	20	0.4448[7]	2.6	80	20	0	0.7499[7]
Stage[3]									
pTa	467	90.1	9.9		2.2	88.7	10.5	0.6	
pT1	247	73.3	26.7		2.5	80.2	15.8	4	
pT1–	39	71.8	28.2		3.6	84.6	10.3	5.1	
pT2–	201	64.2	35.8	< 0.0001	3	73.1	21.4	5.5	< 0.0001[6]
Grade[3]									
G1	123	95.9	4.1		2.1	93.5	5.7	0.8	
G2	470	86.4	13.6		2.3	86.4	11.9	1.7	
G3	365	65.5	34.5	< 0.0001	2.9	75.6	19.7	4.7	< 0.0001
Stage and grade[3]									
pTa G1	123	95.9	4.1		2.1	93.5	5.7	0.8	
pTa G2	298	91.6	8.4		2.2	89.3	10.1	0.7	
pTa G3	46	65.2	34.8		2.7	73.9	26.1	0	
pT1 G2	110	78.2	21.8		2.4	79.1	15.5	5.5	
pT1 G3	137	69.3	30.7		2.7	81	16.1	2.9	
pT2–4 G2	49	75.5	24.5		2.5	83.7	16.3	0	
pT2–4 G3	152	60.5	39.5	< 0.0001	3.1	69.7	23	7.2	< 0.0001

[1]Number of tumors with interpretable results (only first biopsies are considered).–[2]Only pT2–4, not all subtypes, shown.–[3]Only TCC.–[4]Chi-square p-value.–[5]Mean *CMYC* copy number.–[6]pT1–excluded.–[7]TCC *vs.* respective histological type.

Results

CMYC *copy number changes*

A subset of 1,853 tumor samples (only the first biopsy of every patient) was analyzed by FISH for *CMYC* gene copy number. FISH was successful in 1,052 (56.8%). Increased gene copy number state (3 or more than 3 gene copies) was found in 219 tumors (20.8%). The assessment based on the ratio of the gene copy number as compared to the centromere copy number of the same chromosome revealed amplifications in 31 tumors (2.9%) and gains in another 148 tumors (14.1%). The relationship between *CMYC* alterations and tumor phenotype according to both selected definitions are summarized in Table II. Both increased *CMYC* copy numbers and *CMYC* gene amplifications were strongly associated with tumor stage and grade ($p < 0.0001$ each). Increased copy numbers were less frequent in squamous cell carcinomas as compared to urothelial cancers ($p = 0.0373$). This difference was not found, however, for *CMYC* gene amplifications. Comparison of different histologic subtypes was restricted to the subset of 258 muscle invasive (pT2–4) cancers. This was done to avoid a statistical bias because histologic subtypes other than urothelial cancers occur almost exclusively in muscle invasive tumors.

Gene alterations and prognosis

In order to evaluate the prognostic role of *CMYC* gene alterations in transitional cell carcinoma (TCC), tumor-specific survival and tumor progression were selected as clinical endpoints in the subgroup of muscle invasive carcinomas (pT2–4) and in pT1 carcinomas, respectively. Tumor recurrence was used as an endpoint in pTa tumors. The validity of the clinical data attached to the arrayed tissues is demonstrated in a survival analysis including tumor stage and grade (Figs. 1 and 2). Alterations of *CMYC* were linked to reduced patient survival if tumors of all stages and grades were jointly analyzed (amplification *vs.* normal, $p = 0.0281$; increased copy number *vs.* normal copy number, $p = 0.0078$; data not shown). Significance, however, was not maintained if the analysis was restricted to the subgroup of pT2–4 carcinomas (amplification *vs.* normal, $p = 0.4037$; increased copy number *vs.* normal copy number, $p = 0.6300$; Figs. 3 and 4). Likewise, there was no association with *CMYC* alterations and progression in pT1 tumors ($p = 0.6414$; data not shown) or recurrence in pTa tumors ($p = 0.2692$; data not shown).

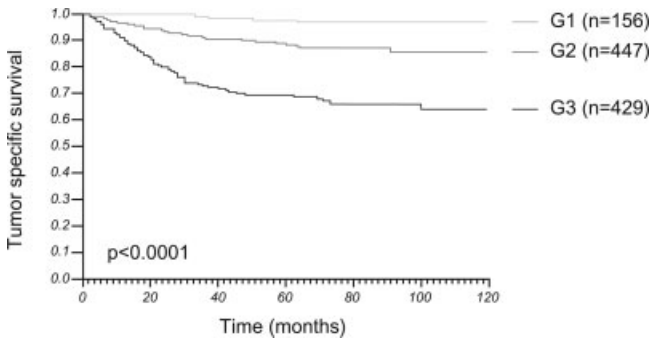

FIGURE 2 – Influence of tumor grade on tumor-specific survival in 1,032 bladder cancer patients (patients with nonurothelial cancers excluded).

Discussion

Our data show a relevant frequency of absolute and relative copy number increases of the *CMYC* gene in urinary bladder cancer that were strongly linked to high grade and advanced stage in bladder cancer. Accordingly, *CMYC* alterations were linked to poor survival if all tumors of all stages were jointly analyzed. To exclude the strong influence of tumor stage on patient survival, analyses of clinical endpoints were restricted to the clinically relevant subgroups of patients, *i.e.*, tumor recurrence in pTa, tumor progression in pT1 and patient survival in pT2–4 cancers. It showed that there was no association with clinical outcome independently from tumor stage.

No generally accepted standards exist for definition of altered gene copy numbers. We therefore used 2 different methods for analyzing the *CMYC* copy number changes. First, we categorized all tumors with less than 3 *CMYC* copy numbers per cell as normal *CMYC* copy number state. Consequently, tumors with 3 or more gene copies were considered to have increased *CMYC* copy number. A total of 30.6% of our invasively growing tumors (pT1–4) had an increased *CMYC* copy number. This number is somewhat lower than reported in some previous FISH studies (50–90%).[20,25] This is probably due to our cautious definition for aberrations

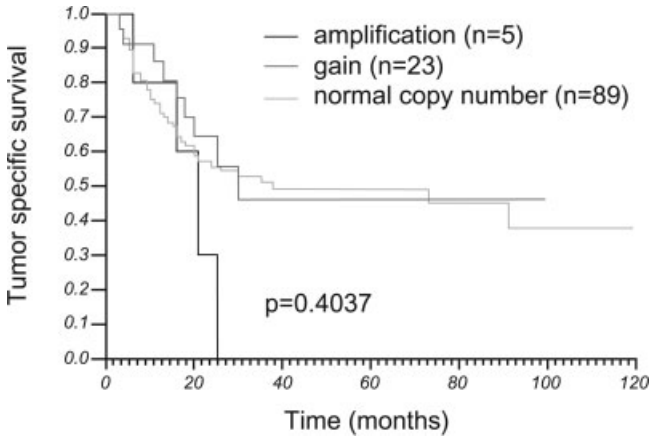

FIGURE 3 – *CMYC* amplifications and gains and tumor-specific survival in pT2–4 TCC.

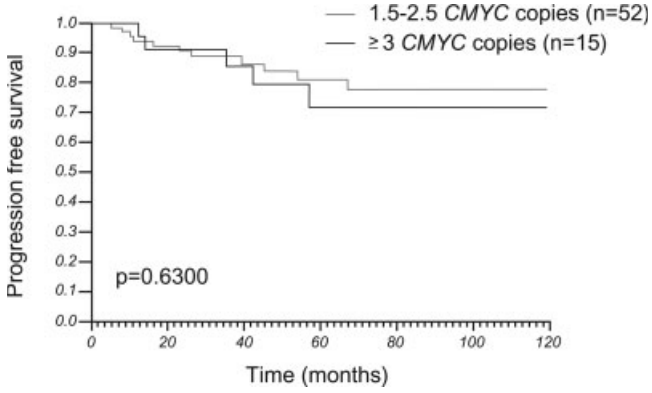

FIGURE 4 – *CMYC* absolute copy number state and prognosis.

(aberration in > 50% of cells). Also, we analyzed partly truncated nuclei on tissue sections while dissociated nuclei were investigated in most previous studies.[20,25]

Another widely accepted method for gene copy number assessment is based on the ratio of the gene copy number as compared to the centromere copy number of the same chromosome. In our second analysis, we therefore used a cutoff of 2.0 for the ratio *CMYC*/centromere 8 to define amplification. This is analogous to the cutoff of the FDA-approved PathVysion kit (Vysis-Abbott, Downers Grove, IL) for detection of HER2 amplification. Our overall amplification rate of 2.9% was comparable to previous studies that had found *CMYC* amplification in 3 out of 87 (3.4%) tumors (24) or in 1 of 37 (3%) tumors.[35] A slightly higher frequency (6.5%) was found in the FISH study of Watters *et al*.[25] and in studies with less quantitative methods such as competitive PCR (7.5%).[17]

Lower levels of relative increases of the *CMYC* gene were recorded as *CMYC* gains in this analysis. Such *CMYC* gains are likely to reflect large but relatively low-level overrepresentations of the entire 8q arm as it is often seen in CGH analysis of bladder cancer and other tumors. However, our frequency of *CMYC* gain (17.7% in pT1–4 cancers) was somewhat lower than the 28–42% found in CGH studies.[36–40] One possible explanation for this discrepancy could be a bias of previous CGH studies toward large tumors with a high content of tumor cells. The higher rate of tumors with an absolute gain of *CMYC* as compared to the frequency of relative gains could also suggest that overrepresentations of *CMYC* may often include a large stretch of the chromosome including centromere 8. In that case, 8q could be overrepresented in CGH analysis without an abnormality in the ratio *CMYC*/centromere 8.

Independent of the definitions used in this study, *CMYC* alterations were strongly associated with high-grade and invasive tumor growth. This fits well with the results of several previous reports finding similar associations of genomic *CMYC* alterations with advanced grade and stage.[20,25] All these data fit well with models suggesting 2 different entities of bladder neoplasms, 1 (pTaG1/G2) being genetically stable with low risk of progression, and the other (pTa/G3 and pT1–4) with a high degree of genetic instability and a high risk of progression.[36,39,41]

Although large-scale descriptive information is provided in this study, no direct evidence is provided for a functional role of *CMYC* in bladder cancer. The strongest argument for an important role of *CMYC* gene alterations in bladder cancer comes from the presence of high-level amplifications in a small fraction (< 3%) of cases. It is likely that such a high-level *CMYC* amplification resulted in a consecutive CMYC overexpression that has provided a direct growth advantage to these cancer cells. The functional relevance of low-level absolute or relative *CMYC* overrepresentations is less clear. We were unable to identify a CMYC antibody that yielded satisfactory results (association of protein expression with high-level amplification) in our hands. This renders it impossible to estimate the clinical impact of CMYC protein expression in bladder cancer since potential epigenetic factors leading to enhanced or decreased *CMYC* transcription activity without detectable gene copy number changes cannot be assessed. Instability of the CMYC protein in formalin-fixed tissues may be a major reason for these difficulties. The inability for reliable CMYC protein measurement on formalin-fixed tissues is also illustrated by the significant discordance of previous IHC studies describing both no association with the tumor stage[14,15,18] and associations with early stage in cancer.[20,21]

Previous studies have shown that low-level overrepresentations of the *CMYC* gene is often part of a complex rearrangement that includes an overrepresentation of a large segment of 8q and often also a deletion of 8p.[20,23,36–45] It is therefore possible that at least in a fraction of bladder tumors, the genomic *CMYC* gene alteration itself, as detected with our assay, may not be the reason for a significant growth advantage of altered cells. It is also possible that cells with a detectable *CMYC* overrepresentation are selected in the tumor, *e.g*., by a growth advantage driven by overexpression of one or multiple other genes on 8q or by inactivation of a tumor suppressor gene on 8p.

In conclusion, the large number of bladder tumors analyzed in our TMA allowed us to obtain a comprehensive picture on the *CMYC* copy number changes in bladder cancer. Overrepresentations of the *CMYC* gene clearly linked to a genetically unstable high-grade and/or invasive tumor phenotype. However, no evidence was found that, within this group of unstable tumors, *CMYC* alterations would distinguish a clinically relevant tumor category. The question for the prognostic impact of CMYC protein expression remains unanswered since no paraffin-suitable antibody was identified in our study. TMAs are highly suited for rapid large-scale surveys comparing molecular features with clinicopathologic parameters.

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Evaluation von potentiellen Zielgenen innerhalb des 6p22.3-Amplikons beim Urothelkarzinom der Harnblase

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Keywords: Bladder Cancer, 6p22, E2F3, NM_017774, CDKAL1, Amplification, Target Gene, FISH, Tissue Microarray

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Running Title: 6p22 Amplifikationen im Harnblasenkarzinom

Abstract

Amplification of 6p22.3 is one of the most frequent chromosomal alterations in high grade and invasive urinary bladder cancer. In order to determine amplification levels of all known genes inside the 1.6 kb core amplicon, we constructed a small tissue microarray (TMA) from 9 primary bladder cancers and 4 bladder cancer cell lines with known 6p22 amplification, and analyzed it with a panel of 16 overlapping FISH probes constructed from bacterial artificial chromosomes (BACs). The highest amplification rates were observed for the transcription factor E2F3 and the adjacent gene NM_017774, the function of which is not known. For a more detailed analysis of these genes, additional large section analysis was done in 19 primary bladder cancers and 18 bladder cancer cell lines. It showed that E2F3 and NM_017774 were always coamplified, but amplification levels in terms of the number of gene copies were slightly higher (16-19 copies per nucleus) for E2F3 as compared to NM_017774 (13-15 gene copies). Our study demonstrates that E2F3 and NM_017774 are located on the top of the 6p22.3 amplicon in bladder cancer. It remains to be studied which one of the two genes drives 6p22 amplification, or if both genes contribute jointly to the aggressive features of 6p22 amplified bladder cancers.

Einleitung

Amplifikationen der chromosomalen Region 6p22 gehören zu den häufigsten genetischen Veränderungen des Harnblasenkarzinoms. Sie treten in etwa 10% aller urothelialen Tumoren auf, insbesondere in invasiv wachsenden (pT1-4) Karzinomen (1). Der genaue Entstehungsmechanismus von Genamplifikationen ist bislang ungeklärt. Es wird vermutet, daß Amplifikationen dazu dienen, die Transkriptionsrate eines oder mehrere Gene massiv zu steigern, um eine natürliche Limitierung der Wachstumsrate durch begrenzt verfügbare Genprodukte zu übergehen (2). So ist zum Beispiel beim Mammakarzinom die Amplifikation des ERBB2 Gens, das für den HER2-Rezeptor (das Zielprotein der Herceptin-Therapie) kodiert, mit einer starken HER2 Proteinüberexpression, Entdifferenzierung, und ungünster Prognose assoziiert (3). Der Erfolg der Herceptin-Therapie beim Mammakarzinom hat aber auch gezeigt, dass amplifizierte Gene die „Achillesferse“ eines Tumors aufzeigen können, und somit potentielle Ziele für neue Genspezifische Therapeutika darstellen (4). Das Zielgen der 6p22-Amplifikation beim Harnblasenkarzinom ist bislang unbekannt. Kartierungen des Amplikons durch Fluoreszenz in situ Hybridisierung (FISH) mit überlappenden Sonden haben gezeigt, dass insgesamt 8 bekannte Gene (PRL, SOX4, NM_017774, E2F3, OACT1, ID4, IBRDC2, DEK) in der 1,5 Megabasen überspannenden amplifizierten Region lokalisiert sind. Allerdings werden die meisten dieser Gene in 6p22 amplifizierten Tumoren entweder gar nicht oder nur in äusserst geringen Mengen exprimiert, sodaß sie als Zielgene nicht in Frage kommen (5-7). Lediglich für zwei Gene konnte bislang ein Zusammenhang zwischen Genamplifikation und verstärkter Expression festgestellt werden (1, 5). Eines dieser Gene ist der Transkriptionsfaktor E2F3, ein Mitglied einer Gruppe von Genen (E2Fs) mit zentraler Funktion bei der Kontrolle des Zellzyklus. E2F3 ist in seiner inaktiven Form an das Retinoblastoma-Protein (Rb) gebunden. Phosphorylierung von Rb durch zyklinabhängige Kinasen (CDKs) führt zur Dissoziation des Rb/E2F3-Komplexes und zum Übergang der Zelle von der G1- in die S-Phase des Zellzyklus (8). Untersuchungen der E2F3 Amplifikation und Expression an einem Gewebemikroarray (TMA) mit über

2300 Tumoren haben ergeben, das E2F3-Veränderungen mit invasivem Wachstum, fortgeschrittenem Tumorstadium, schlechter Differenzierung, und erhöhter Proliferationsrate einhergehen (1). Das zweite Gen, mit der Bezeichung NM_017774, grenzt direkt an E2F3. Die Funktion von NM_017774 ist zwar noch unbekannt, doch deuten bestimmte funktionelle Motive der Aminosäurekette darauf hin, dass es sich um ein Protein handeln könnte, das bei der Translation von der mRNA zum Protein eine wichtige Rolle spielen könnte (9). Somit kommt neben E2F3 auch NM_017774 als Kandidat für das Zielgen der 6p22-Amplifikation in Frage. Hinweise darauf, welchem der beiden Gene die Ampifikation bevorzugt gilt, könnten zum einen die Häufigkeit der Amplifikation, zum anderen die Amplifikationsstärke geben. Das Ziel unserer Studie war daher, die Amplifikationsstärke und Häufigkeit insbesondere von E2F3 und NM_017774, sowie weiterer benachbarter Gene im 6p22.3 Amplikon, zu untersuchen.

Material und Methoden

Studiengewebe

19 Harnblasenkarzinome mit 6p22-Amplifikation wurden in einer früheren Studie am TMA (1) identifiziert. Für die hier vorliegende Studie wurden Großschnitte jener Tumoren untersucht. Eine Übersicht der verwendeten Gewebeproben mit histo-pathologischen Parametern ist in Tabelle 1 dargestellt. Zur Ergänzung wurde außerdem ein bereits existierender Zelllinien-TMA mit 18 verschiedenen Blasenkarzinom-Zelllinien (10) auf E2F3 und NM_017774-Veränderungen untersucht. Folgende Zelllinien sind auf dem TMA vertreten: J82, RT4, T24, HT1376, SW780, TCC SUP, Hs769.T, Hs853.T, HB-CLS-1, HB-CLS-439, RT112, RT112 D21, EJ28, 5637, BFTC905, Ku1919, SCaBER und CRL7930. Darüber hinaus wurde ein spezieller TMA (6p22-TMA) aus 4 Zelllinien (HT1376, TCC SUP, RT112-D21, 5637) und 9 Primärtumoren mit 6p22 Amplifikation hergestellt, um die Amplifikationsstärke der Gene im Amplikon zu vergleichen.

Fluoreszenz in situ Hybridisierung (FISH)

Zur FISH-Untersuchung wurden DNS-Sonden aus den künstlichen Bakterienchromosomen (BACs/PACs) PAC dJ177P22 (für E2F3; Sanger Centre, UK) und BAC RP3444C7 (für NM_017774; RZPD, Berlin, Germany) hergestellt. Die weiteren, für diese Studie verwendeten BACs, sind in Abbildung 1 dargestellt. Alle BACs/PACs wurden über Nacht in LB Medium bei Raumtemperatur inkubiert. Die Plasmide mit den humanen DNS Sequenzen wurden mit einem kommerziell erhältlichen Kit (Miniprep, Qiagen, Hilden) den Herstellervorschriften entsprechend extrahiert. Beide Sonden wurden über ein Standard-Nicktranlationsverfahren mit Digoxigenein markiert. Als interne Referenz wurde für jede Hybridisierung eine Spektrum-Rot markierte Sonde gegen Zentromer 6 ko-hybridisiert (Vysis, Downers Grove, IL). Hybridisierung und stringente Waschungen wurden exakt nach dem Vysis-Protokoll (LSI Prozedur) durchgeführt. Die Sonden gegen E2F3 und NM_017774 und die anderen BACs/PACs wurden durch Inkubation mit einem Primärantikörper gegen Digoxigenin (Maus anti Dig, 45 min, 37°C) und FITC-markierten Sekundärantikörper (Ziege anti Maus, 45 min, 37°C) nachgewiesen. Alle Präparate wurden unter einem Fluoreszenzmikroskop mit geeigneten Filtern von einer Person (HN) ausgewertet. Für jedes Gewebe wurde die Anzahl der FISH-Signale für Zentromer 6 und E2F3, sowie NM_017774 in ca. 20 Zellkernen ausgezählt.

Ergebnisse

Amplifikationsstärke der Gene im 6p22.3 Kernamplikon

16 überlappende BAC/PAC Sonden wurden auf dem 6p22-TMA hybridisiert. Die durchschnittliche gezählte Kopiezahl (4 Zelllinien, 9 Primärtumoren) je Sonde ist in Abbildung 1 dargestellt. Die weitaus höchsten Kopiezahlen (16-18 Gensignale) wurden für E2F3 und einen ca 400 Kb grossen DNS Abschnitt um E2F3 herum gefunden. Das benachbarte NM_017774 Gen weist nur im 5'-Bereich eine solch hohe Kopiezahl auf. Die weiter 3' gelegenen Exons des Genes zeigten hingegen nur eine „durchschnittliche" Amplifikationsstärke, wie

sie auch für andere bekannte Gene im Kernamplikon (z.B. ID4, SOX4) gefunden wurden.

Ko-Amplifikationsmuster von E2F3 und NM_017774

Um ein genaueres Bild des Amplifikationsmusters im Zentrum des Amplikons zu erhalten, wurde die FISH Analyse für E2F3 und NM_017774 in einer Serie von 19 Primärtumoren an Standard-Gewebeschnitten, sowie einem Blasentumor-Zelllinien TMA mit 18 Zelllinien durchgeführt. Die FISH Ergebnisse sind in Tabelle 1 zusammengefasst. Der Vergleich zwischen E2F3 und NM_017774 zeigt, dass alle E2F3 amplifizierten Tumoren und Zelllinien ebenfalls NM_017774 Amplifikationen aufweisen. Es konnte kein einzelner Fall identifiziert werden, in dem nur E2F3, aber nicht NM_017774 (oder umgekehrt) amplifiziert vorlagen. Auch die Unterschiede in der Genkopiezahl waren in allen Fällen nur äußerst minimal.

Diskussion

In einer vorhergehenden Studie konnten wir zeigen, dass Amplifikationen der chromosomalen Region 6p22.3 mit invasivem Wachstum, fortgeschrittenem Tumorstadium, Entdifferenzierung und erhöhter Proliferationsrate assoziiert sind (1). Obwohl das 6p22.3 Amplikon mit ca. 1,6 Kb relativ klein ist, umschließt es doch mindestens 8 Gene. Welche(s) dieser Gene letztendlich das Ziel der Amplifikation ist, konnte bislang nicht geklärt werden.

Bisherige Untersuchungen haben gezeigt, dass 6p22.3 Amplifikationen lediglich bei E2F3 und NM_017774 zu einem massiv erhöhten Transkriptionsniveau führen. Unsere Untersuchung des 1,6 Mb umfassenden Kernamplikons stellt eine lückenlose Feinkartierung der Genkopiezahl von 16 Genloci innerhalb dieses Areals dar, mit einer Auflösung von durchschnittlich nur 100 Kb. Das Amplifikationsniveau zeigt sich dabei relativ konstant bei 13-

14 Genkopien, wobei nur 4 Sonden direkt um E2F3 herum eine höhere Kopiezahl (16-19 Signale) erreichen. Diese Zählungen können zwar nur eine grobe Annäherung der tatsächlichen Kopiezahlen wiedergeben, weil eine exakte Bestimmung der Signalzahl bei Genamplifikationen aufgrund von Signalclustern und überlappenden Signalen praktisch unmöglich ist. Dennoch weist der E2F3-Locus das vergleichsweise höchste Amplifikationsniveau auf. Das benachbarte NM_017774 Gen dagegen ist nicht über seine gesamte Länge gleichmässig stark amplifiziert. Eine mit E2F3 vergleichbar hohe Kopiezahl ist nur im Bereich der vorderen (3' gelegenen) 3-5 Exons zu finden. NM_017774 ist mit 1,74 Kb ein zwar kein übermäßig großes Gen, jedoch erstrecken sich seine 14 Exons über eine genomische Distanz von 685 Kb. Da für die Transkription des gesamten Genes nur ein zusammenhängendes Stück genomischer DNS als Matrize in Frage kommt, muss die geringere Kopienzahl der hinteren (5') Exons als limitierend für das Gesamtexpressionsniveau angesehen werden. Dies würde bedeuten, dass die Amplifikation in erster Linie der Expressionssteigerung von E2F3 zugute kommt, und vielleicht weniger effizient für NM_017774 sein könnte. Allerdings modulieren auch andere Faktoren als die reine Gendosis das Expressionsniveau, z.B. die Verteilung von regulativen Elementen (Enhancer, Silencer) im genomischen Umfeld des Genes, die Verfügbarkeit von Transkriptionsfaktoren, sowie epigenetische Faktoren wie z.B. Transkriptionsregulierung durch Promoter-Methylierung.

Ein weiterer wichtiger Hinweis auf die relative Bedeutung von E2F3 und NM_017774 für die 6p22 Amplifikation kann aus der molekularen Epidemiologie dieser Gene gewonnen werden. Es ist logisch anzunehmen, dass das Zielgen in jedem einzelnen 6p22 amplifizierten Tumor auch von der Amplifikation betroffen sein sollte. Die Entdeckung eines einzigen 6p22 amplifizierten Tumors mit Beteiligung nur des einen Genes – ohne Amplifikation des anderen – wäre folglich ein wichtiges Indiz dafür, dass es sich beim einzeln amplifizierten Gen um das Zielgen der Amplifikation handeln müsste. Obwohl etwa ein Viertel aller E2F3 amplifizierten Tumoren, die in unserer vorhergehenden TMA Analyse als E2F3 amplifiziert gefunden wurden (1) zur Verfügung standen und am Grosschnitt nochmals auf E2F3-

und NM_017774-Amplifikationen nachuntersucht werden konnten, ist in keinem dieser 19 Fälle nur eines der beiden Gene allein amplifiziert gewesen. Dieses Ergebnis lässt vermuten, dass E2F3 und NM_017774 stets ko-amplifiziert vorliegen. Ob dies lediglich auf ihre räumlich Nähe im Genom (<100 Kb Abstand) zurückzuführen ist, oder ob das 6p22-Amplikon möglicherweise über zwei gleichwertige Zielgene verfügt, werden erst funktionelle Untersuchungen zeigen können.

Zusammenfassend ist zu sagen, dass E2F3 und NM_017774 das Zentrum der 6p22 Amplifikation beim Harnblasenkarzinom repräsentieren. Es scheint nicht wahrscheinlich, dass andere Gene in der Region, wie z.B. ID4 oder SOX4, der Amplifikation zu Grunde liegen. Allerdings kann die Untersuchung des Amplifikationsstatus' alleine nicht klären, ob E2F3 oder NM_017774 das wahrscheinlichere Zielgen der 6p22.3 Amplifikation sind. Beide Gene sind vermutlich schon alleine aufgrund ihrer räumlichen Nähe grundsätzlich ko-amplifiziert. Lediglich das etwas höhere Amplifikationsniveau von E2F3 im Vergleich zu NM_017774 könnte ein Hinweis auf eine bedeutendere Rolle von E2F3 sein. Alternativ ist jedoch auch nicht auszuschließen, das beide Gene einen kooperativen Effekt auf das Wachstum 6p22 amplifizierter Tumoren ausüben, wie dies z.B. für c-myc und Her2/neu beim Mammakarzinom beschrieben worden ist (11, 12). Weiterführende funktionelle Analysen, etwa an Blasenkarzinomzelllinien mit und ohne 6p22.3 Amplifikation sind notwendig, um die Frage nach dem Zielgen abschließend zu beurteilen.

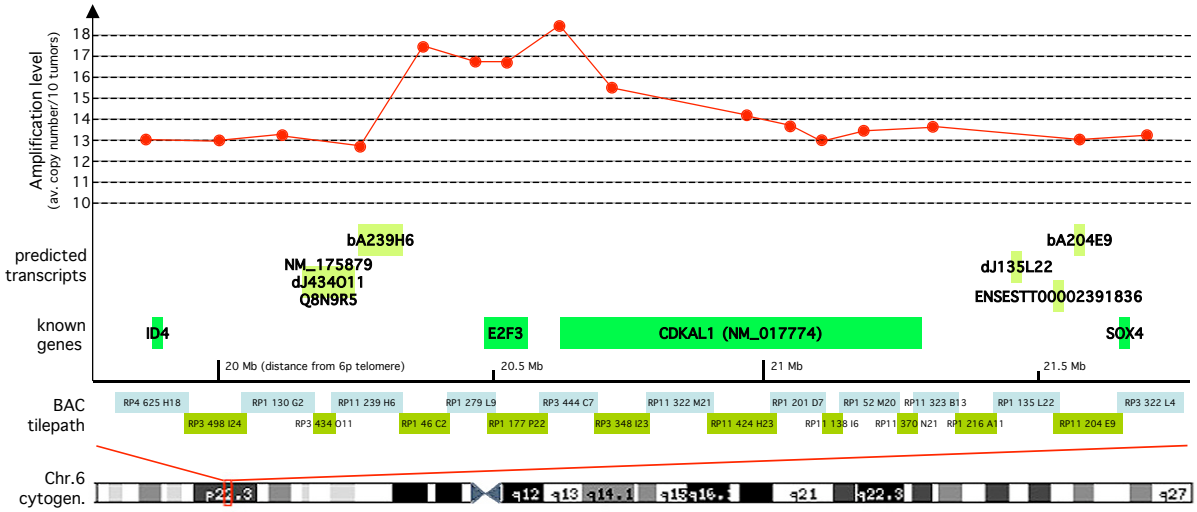
Abbildungen / Tabellen

Tabelle 1: **Ko-Amplifikationsmuster von E2F3 und NM_017774.**

PT=Primärtumor, ZL = Zelllinie

Gewebe	Probe	Stadium/Grad	#C6	#E2F3	#NM_017774	Ergebnis E2F3	Ergebnis NM_017774
PT1	B77.20282	pT2-4/G3	Keine Signale	-	-	-	-
PT2	81/15573	pT1/G3	4-8	4-12	4-12	gain	gain
PT3	B82.2514	pT1/G3	2-4	2-4	2-4	normal	normal
PT4	83/19350	pTa/G3	2-10	10-20	10-20	gain	gain
PT5	84/6859	pT1/G3	1-2	10-20	10-20	amp	amp
PT6	86/18588	pT1/G3	2-4	10-20	10-20	amp	amp
PT7	87/3359	pT1/G3	2-4	10-30	10-30	amp	amp
PT8	87/6655 II	pT2-4/G3	4	10-20	20	amp	amp
PT9	88/32460	pT2-4/G3	2-4	10-20	10-20	amp	amp
PT10	B89.13845	pT1/G3	Keine Signale.	-	-	-	-
PT11	mü91.1770	pT1/G3	2-3	2-3	2-3	normal	normal
PT12	B91.2252	pT1/G3	2-4	6-10	6-10	gain	gain
PT13	92/14878 IA	pT2-4/G3	2-6	50	50	amp	amp
PT14	92/17791	pT2-4/G3	2-4	10	10	amp	amp
PT15	92/22075	pT2-4/G3	2-5	2-8	2-6	gain	gain
PT16	92/8711	pT1/G3	2-4	30	30	amp	amp
PT17	94/1684	pT2-4/G3	2-6	2-10	2-6	normal	normal
PT18	95/11037	pT2-4/G3	2	10	10	amp	amp
PT19	B96.17168	pT1/G3	2-4	2-4	2-4	normal	normal
ZL1	J82	-	2-4	2-6	2-7	normal	normal
ZL2	RT4	-	4	4	5	normal	normal
ZL3	T24	-	2-3	2-6	2-3	normal	normal
ZL4	HT1376	-	2-6	20	20	amp	amp
ZL5	SW780	-	2-4	2-4	2-5	normal	normal
ZL6	TCC SUP	-	2-4	20	20	amp	amp
ZL7	Hs769.T	-	2-4	2-4	2-4	normal	normal
ZL8	Hs853.T	-	2-4	2-4	2-4	normal	normal
ZL9	HB-CLS-1	-	2	2	3	normal	normal
ZL10	HB-CLS-439	-	1	6	4-6	amp	amp
ZL11	RT112	-	2-4	2-4	2	normal	normal
ZL12	RT112 D21	-	2-4	2-6	2-4	normal	normal
ZL13	EJ28	-	2-4	2-4	2-4	normal	normal
ZL14	5637	-	2	20	20	amp	amp
ZL15	BFTC905	-	2-4	2-4	2	normal	normal
ZL16	Ku1919	-	2-4	2-4	2	normal	normal
ZL17	SCaBER	-	4	4	2-4	normal	normal
ZL18	CRL7930	-	4	4	2-4	normal	normal

Abbildung 1: **Schematische Darstellung vom Kerngebiet des 6p22.3 Amplikons:** Überlappende BAC Klone sind am unteren Rand des Diagramms aufgeführt. Die grüne Farbe steht für bereits bekannte Gene, cDNS Transkripte sind hingegen mit hellgrün markiert. Die mittlere Amplifikationsrate (=durchschnittliche Signalintensität in 10 amplifizierten Primärtumoren und 2 amplifizierten Tumorzelllinien) für jeden untersuchten BAC Klon wurde mit einem roten Punkt dargestellt.



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E2F3 is the main target gene of the 6p22 amplicon with high specificity for human bladder cancer

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Amplification of 6p22 occurs in about 10–20% of bladder cancers and is associated with enhanced tumour cell proliferation. Candidate target genes for the 6p22 amplicon include E2F3 and the adjacent gene NM_017774. To clarify which gene is representing the main target, we compared the prevalence of the amplification and the functional role of both genes. Amplification of E2F3 and NM_017774 was analysed by fluorescence *in situ* hybridization on a bladder cancer tissue microarray composed of 2317 cancer samples. Both genes showed amplification in 104 of 893 (11.6%) interpretable tumours and were exclusively found co-amplified. Additional gene expression analysis by real-time polymerase chain reaction in 12 tumour-derived cell lines revealed that amplification of 6p22 was always associated with co-overexpression of E2F3 and NM_017774. Furthermore, RNA interference was used to study the influence of reduced gene expression on cell growth. In tumour cells with and without the 6p22 amplicon, knockdown of E2F3 always lead to unequivocal reduction of proliferation, whereas knockdown of NM_017774 was only capable to slow down cell proliferation in non-amplified cells. Our findings point out that E2F3 but not NM_017774 is driving enhanced proliferation of 6p22 amplified tumour cells. We conclude that E2F3 must be responsible for the growth advantage of 6p22 amplified bladder cancer cells.
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Amplification of the chromosomal region 6p22 is one of the most frequent genetic alterations in urinary bladder cancer, affecting up to 20% of high grade, invasively growing tumours (Bruch *et al*., 2000; Tomovska *et al*., 2001; Hurst *et al*., 2004; Oeggerli *et al*., 2004). In a recent study, we narrowed down the amplicon to a region spanning approximately 1.6 megabases at 6p22.3 enclosing 13 different genes (Tomovska *et al*., 2001). Only two of these genes, the transcription factor E2F3 and the adjacent gene NM_017774, are consistently overexpressed in 6p22.3 amplified bladder cancers and are therefore considered candidate genes driving the amplification (Bruch *et al*., 2000; Feber *et al*., 2004; Hurst *et al*., 2004).

E2Fs play an important role in the retinoblastoma (Rb) pathway (Hunter and Pines, 1994). The regulatory function of Rb largely depends on the ability to bind and inhibit E2F family members of transcription factors including E2F3 (Hiebert *et al*., 1992; Qian *et al*., 1992). We have recently shown that amplification of the E2F3 gene locus is associated with protein overexpression, invasive tumour growth and enhanced cell proliferation (Oeggerli *et al*., 2004). The function of NM_017774 is currently not known, but it shows some homology to a protein that is associated with cyclin–dependent kinase 5 (CDK5RAP1), which is the reason why it has originally been termed cyclin–dependent kinase 5–associated protein 1–like 1. However, it is important to note that there is no experimental evidence for any functional similarities between these two proteins.

In order to determine, whether E2F3 or NM_017774 is the main amplification target, or if both genes might contribute jointly to the aggressive features of 6p22.3–amplified bladder cancers, we first inspected amplification frequencies in 18 bladder cancer cell lines by fluorescence *in situ* hybridization (FISH), as described previously (Wagner *et al*., 1997). We utilized digoxigenated BAC (NM_017774: BAC RP3444C7, RZPD, Berlin, Germany) and PAC (E2F3: PAC dJ177P22, Sanger Centre, Cambridge, UK) probes containing the target genes and a Spectrum Red-labeled chromosome 6 centromeric probe (CEP6) as a reference (Vysis, Downers Grove, IL, USA). Amplifications were found in four of 18 (22%) bladder cancer cell lines (HTB-5, HTB-9, CRL-1472 and HB-CLS-439), showing that E2F3 and NM_017774 were always co-amplified.

A bladder cancer prognosis tissue microarray (TMA), composed of 2317 formalin-fixed paraffin–embedded tissues (Oeggerli *et al*., 2004), was then used to comprehensively compare the amplification frequencies of both genes. We hypothesized that the main amplification target would be present in all tumours that reveal

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amplification of the 6p22.3 genomic region. Initial amplification frequencies were 9.8% for NM_017774 and 11.4% for E2F3. A small subset of 34 tumours (3.8%) could be identified, exhibiting amplification of only one gene (see Table 1a and Table 1b). The following case-by-case comparison of these tumours using conventional large tissue sections demonstrated, however, that every tumour with E2F3 amplification had also NM_017774 amplification, and vice versa. The initially observed discrepancies were either due to variable interpretation of borderline findings in low level amplified tumours (15 cases) or counting errors because of low FISH signal intensities, high background, tissue damage or technical artefacts (19 cases). In summary, co-amplification of E2F3 and NM_017774 was found in all 6p22.3 amplified tumours (11.6%). As a consequence, amplification of NM_017774 is identically associated with invasive and high-grade phenotype, and patient prognosis as already published for E2F3 (Oeggerli *et al*., 2004).

As FISH analysis could not identify either E2F3 or NM_017774 as the primary amplification target, we next performed mRNA expression analysis. We expected that the main amplification target gene would show a particularly strong mRNA expression increase. Because of the superior RNA quality in freshly collected tissues as compared to formalin-fixed paraffin–embedded samples, we compared mRNA levels of three amplified and four non-amplified bladder cancer cell lines. Detailed results of our gene expression analysis can be taken from Figure 1. E2F3 was generally expressed at higher levels than NM_017774 (average difference 4.25-fold). However, 6p22.3 amplification had a comparable influence on E2F3 and NM_017774 expression, scaling up individual mRNA levels at least 10-fold. These findings are in line with a previous analysis in cell lines HTB-5, HTB-9, JO'N and CRL-1472 by Hurst *et al*. (2004) who also found significantly increased expression of NM_017774 and E2F3 following the amplification of 6p22.3.

These results point to the hypothesis that both genes might jointly contribute to the aggressive features of 6p22.3–amplified bladder cancers. Clearly, DNA amplification is a perfect method to co-overexpress neighbouring genes. Evidence of clusters of co-overexpressed genes have already been detected in human, fly and worm (Wang *et al*., 1995). Examples in humans include the non-I-integrin alpha-chain genes located in clusters on chromosomes 2, 12 and 17. It has been suggested before that keeping functionally related genes

Table 1a Initial FISH analysis for E2F3 and NM_017774

	Sample	*Stage/grade*	*Gene copy number*			*Ratio*		*Initial score*
			C6	*E2F3*	*NM_017774*	*E2F3*	*NM_017774*	
1	PT1	PT1/G3	6	15	20	2.5	3.3	Borderline
2	PT2	PT1/G3	4	10	12	2.5	3.0	Borderline
3	PT3	pT1/G3	2–4	8	15	2.7	5.0	Borderline
4	PT4	pT2–4/G2	2–4	8	10	2.7	3.3	Borderline
5	PT5	pTa/G2	1–2	4	5	2.7	3.3	Borderline
6	PT6	pT2–4/G3	2–5	10	15	2.9	4.3	Borderline
7	PT7	pT2–4/G3	2–5	10	12	2.9	3.4	Borderline
8	PT8	PT1/G3	2	6	5	3.0	2.5	Borderline
9	PT9	PT1/G2	1	3	2	3.0	2.0	Borderline
10	PT10	PT1/G3	4–6	15	10	3.0	2.0	Borderline
11	PT11	pT2–4/G3	2–5	12	10	3.4	2.9	Borderline
12	PT12	PT1/G3	1–2	6	4	4.0	2.7	Borderline
13	PT13	pTa/G3	2	8	5	4.0	2.5	Borderline
14	PT14	pT2–4/G3	1	4	2	4.0	2.0	Borderline
15	PT15	pT2–4/G3	1	4	2	4.0	2.0	Borderline
16	PT16	pT1/G3	2–4	10	2	3.3	0.7	Discrepant
17	PT17	pT1/G3	2	10	2	5.0	1.0	Discrepant
18	PT18	pT1/G3	2–5	3	13	0.9	3.7	Discrepant
19	PT19	pT2–4/G3	2	12	2	6.0	1.0	Discrepant
20	PT20	pT1/G3	4	12	2	3.0	0.5	Discrepant
21	PT21	pT2–4/G3	2–5	6	15	1.7	4.3	Discrepant
22	PT22	pTa/G3	2	15	2	7.5	1.0	Discrepant
23	PT23	pT1/G3	2–4	10	2	3.3	0.7	Discrepant
24	PT24	pT2–4/G3	2–3	10	2	4.0	0.8	Discrepant
25	PT25	pT2–4/G3	2–3	2	20	0.8	8.0	Discrepant
26	PT26	pT2–4/G3	2–4	10	2	3.3	0.7	Discrepant
27	PT27	pT1/G3	2–3	2	35	0.8	14.0	Discrepant
28	PT28	pT2–4/G3	2–4	15	2	5.0	0.7	Discrepant
29	PT29	pT1/G3	2	15	2	7.5	1.0	Discrepant
30	PT30	pT1/G3	1–2	2	15	1.3	10.0	Discrepant
31	PT31	pT1/G3	2–4	4	10	1.3	3.3	Discrepant
32	PT32	pT1/G3	1–2	2	30	1.3	20.0	Discrepant
33	PT33	pT2–4/G3	2	20	2	10.0	1.0	Discrepant
34	PT34	pT1/G3	1	20	4	20.0	4.0	Discrepant

Abbreviations: FISH, fluorescence *in situ* hybridization; PT, primary tumour.

Table 1b Large section FISH analysis for E2F3 and NM_017774

	Sample	*Stage/grade*	*Gene copy number*			*Ratio*		*Final score*
			C6	*E2F3*	*NM_017774*	*E2F3*	*NM_017774*	
1	PT16	pT1/G3	2–3	2–3	2–3	1.0	1.0	Normal
2	PT17	pT1/G3	2–4	2–4	2–4	1.0	1.0	Normal
3	PT18	pT1/G3	2–4	2–4	2–4	1.0	1.0	Normal
4	PT19	pT2–/G3	2–5	2–8	2–6	1.4	1.1	Gain
5	PT20	pT1/G3	4–8	4–12	4–12	1.3	1.3	Gain
6	PT21	pT2–/G3	2–6	2–10	2–6	1.5	1.5	Gain
7	PT22	pTa/G3	2–10	10–20	10–20	2.5	2.5	Gain
8	PT23	pT1/G3	2–4	6–10	6–10	2.7	2.7	Gain
9	PT24	pT2–/G3	2–4	10	10	3.3	3.3	Co-amplified
10	PT25	pT2–/G3	4	10–20	20	3.8	5.0	Co-amplified
11	PT26	pT2–/G3	2	10	10	5.0	5.0	Co-amplified
12	PT27	pT1/G3	2–4	10–20	10–20	5.0	5.0	Co-amplified
13	PT28	pT2–/G3	2–4	10–20	10–20	5.0	5.0	Co-amplified
14	PT29	pT1/G3	2–4	10–30	10–30	6.7	6.7	Co-amplified
15	PT30	pT1/G3	1–2	10–20	10–20	10.0	10.0	Co-amplified
16	PT31	pT1/G3	2–4	30	30	10.0	10.0	Co-amplified
17	PT32	pT2–/G3	2–6	50	50	16.7	16.7	Co-amplified
18	PT33	pT2–/G3	Tissue[a]	—	—	—	—	—
19	PT34	pT1/G3	Tissue[b]	—	—	—	—	—

Abbreviations: FISH, fluorescence *in situ* hybridization; PT, primary tumour. [a]Tissue = insufficient tissue left. [b]Tissue = tissue damage.

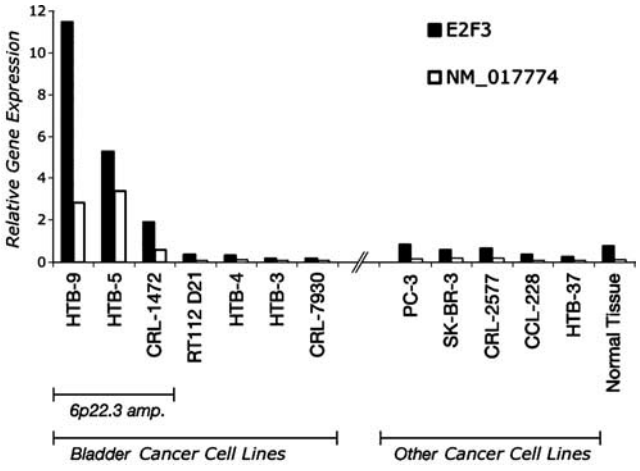

Figure 1 Relative gene expression levels of E2F3 and NM_017774 in various cancer cell lines with and without 6p22.3 amplification. Both genes are markedly upregulated in amplified cell lines. Cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown under standard cell culturing conditions. RNA isolations were carried out according to the manufacturer's specifications using DNase I system in combination with the RNeasy kit (Qiagen, Hilden, Germany). RNA concentrations were determined with a spectrophotometer. For each cell line, 250 ng total RNA was used as starting material for complementary DNA (cDNA) synthesis combined with Oligo-dT (Roche, Basel, Switzerland) as primer. Real-time PCR was performed in duplicates in 20 μl reactions containing: 2 μl cDNA template (from 1:2 dilutions of cDNA synthesis reaction), 10 μl FastStart SYBR Green I PCR Master Mix (Roche), $MgCl_2$ as well as forward and reverse primer mix (10 mM each). Thermal cycling conditions for the LightCycler Instrument (Roche) were: one cycle at 95°C for 10 min at steps of 20°C/s (activation), 40 cycles at 95°C for 15 s at 20°C/s, 55°C for 10 s at 20°C/s and 72°C for 10 s at 5°C/s (amplification) and one additional cycle at 95°C for 1 s at 20°C/s, 65°C for 15 s at 20°C/s and 99°C for 1 s at 0.05°C/s (melting). Relative levels of expression were determined using the $2^{-\Delta\Delta C_T}$ method as described by Livak and Schmittgen (2001). All samples were normalized against glyceraldehydes-3-phosphate dehydrogenase (G3PDH).

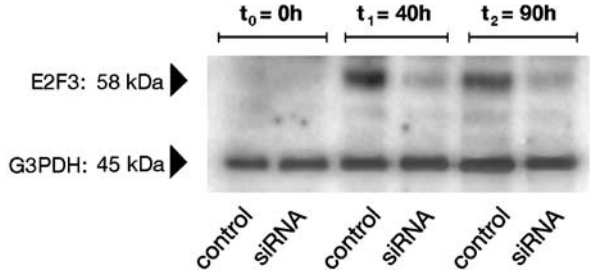

Figure 2 Western blot analysis displaying the reduction of E2F3 expression, induced by E2F3-specific knockdown in 6p22.3-amplified cancer cell line HTB-5. Nonsense RNAi was used as negative control, G3PDH as loading control. Cells were serum starved at the beginning of the experiment (at that time E2F3 is not expressed). Protein was extracted from cell line HTB-5, according to Leone *et al*. (1998). Ten micrograms protein of each sample was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis for reduced samples on 10% polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Blots were incubated with mouse monoclonal E2F3 Ab-4 primary antibody (1:1000) (Lab Vision, Fremont, CA, USA) followed by incubation with goat anti-mouse IgG secondary antibody (1:2000) (Fc, AP127P; Juro Supply AG, Lucerne, Switzerland). Finally, blots were processed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Duebendorf, Switzerland) and exposed to Kodak AR film (Stuttgart, Germany).

near could be advantageous for a cell because it may ease the burden of unpacking of DNA for transcription (Lee and Sonnhammer, 2003). It appears possible that amplification might not always target only one particular gene, but two or more genes that contribute to a common function or pathway. Although only little is known about the possible function of NM_017774, the presence of particular functional domains in the predicted protein structure have linked it to the protein translation machinery (Altschul *et al*., 1997). It can be expected that such a cooperative effect of E2F3 and

NM_017774 would result in a particular strong growth advantage and that any reduction in the quantity of one of these two genes should be sufficient to reverse the effect.

In order to test this hypothesis, we decided to perform gene silencing experiments in 6p22.3–amplified cell lines. RNA interference (RNAi) is an established method to specifically inactivate mRNA of selected target genes (Elbashir *et al*., 2001; Paddison *et al*., 2002). Two 6p22.3–amplified (HTB-5, CRL-1472) and two non-amplified cell lines (CRL-7930, PC-3) were tested for their suitability for RNAi treatment. SYBR Green real-time polymerase chain reaction (PCR) (LightCycler, Roche, Basel, Switzerland) was employed to measure the effect of RNAi on target gene expression.

Applying this technique resulted in an always more than 50% decrease of mRNA levels for both potential target genes over a period, starting from 12 h after transfection and lasting until the end of the experiments (after 6 days). Based on these studies, non-amplified cell line CRL-7930 and amplified cell line HTB-5 were selected for subsequent experiments.

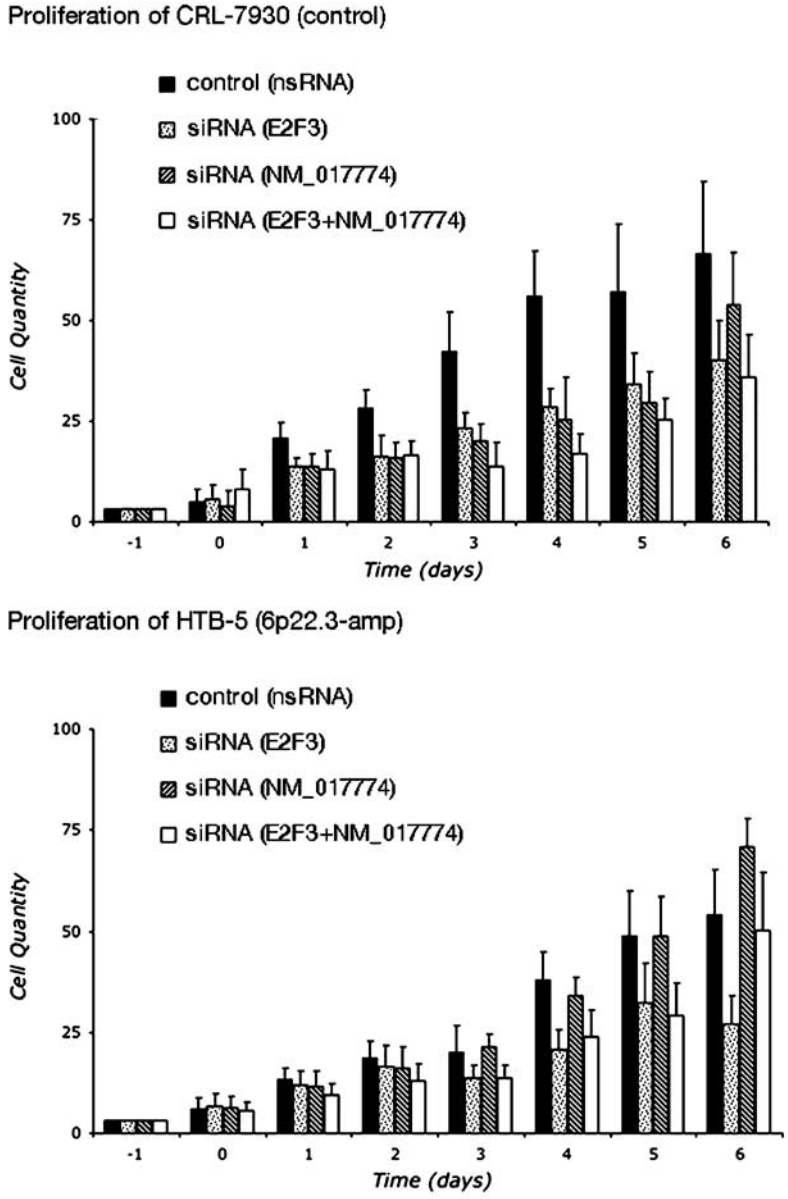

Figure 3 Cell proliferation of bladder cancer cell lines with and without 6p22.3 amplification. Bars illustrate observed differences in cell proliferation rates following gene-specific and combined siRNA treatment against both amplification targets (E2F3 and NM_017774). Silencing of E2F3 always markedly decreased cell proliferation, whereas silencing of NM_017774 only inhibited non-amplified CRL-7930 cells (top), but failed to affect proliferation in amplified HTB-5 cells (bottom). Controls, treated with nonsense RNAi, are shown in black. To monitor the effect of gene silencing on tumour cell proliferation, growth curves were calculated from siRNA-treated and untreated cell cultures. Replicate cultures were grown in parallel allowing for repeated cell harvesting and counting in 24 h intervals. All utilized specific siRNA sequences are available on request. Cell counting was performed using a 'Neubauer' counting chamber. Standard counting procedures were followed to determine cell quantity (Lindl TaB, 1989).

In cell line CRL-7930, mRNA levels decreased until day 4 after transfection and did not rise again until the end of the experiment (day 6). Knockdown levels were slightly higher for E2F3 (73% decrease of mRNA level; average from days 4 to 6) as compared to NM_017774 (59% decrease of mRNA level; average from days 4 to 6; $P=0.0016$).

In the amplified cell line HTB-5, the lowest mRNA expression levels were reached already 24 h after transfection. No difference between E2F3 (53%) and NM_017774 (50%) was detectable ($P=0.4186$, average knockdown from days 1 to 4). Combined knockdown did not result in a further decrease of individual mRNA levels as compared to separate knockdown, in all tested cell lines. Decreased protein expression of E2F3 in HTB-5, induced by E2F3-specific siRNA, was additionally confirmed by Western blot analysis and results are visualized in Figure 2. The silencing power of NM_017774-specific siRNA could not yet be documented by Western blot, because NM_017774-specific antibodies are currently not available.

The effects of E2F3, NM_017774 and combined gene knockdown on cell proliferation are compared against the effect of nonsense siRNA control and are shown in Figure 3: In the non-amplified cell line (CRL-7930), knockdown of E2F3 as well as NM_017774 resulted in a pronounced decrease of the cell proliferation rate (average over 6 days: E2F3: −43.1%, $P=0.004$; NM_017774: −48.7%, $P=0.006$; maximum E2F3: −48.2% at day 4; maximum NM_017774: −55.4% at day 4). Simultaneous knockdown exerted even a stronger proliferation decrease amounting to −57.9% ($P=0.008$; maximum −69.6% at day 4).

In the amplified cell line (HTB-5), knockdown of E2F3 resulted in a comparable decrease of the cell proliferation, like it had been observed in non-amplified cells (average: −36.7%, $P=0.018$; maximum −44.7% at day 4). In contrast, knockdown of NM_017774 had no negative influence on cell proliferation in amplified cells (average: +5%, $P=0.309$; maximum –10.9% at day 4). The combined knockdown (average: −27.7%, $P=0.011$; maximum −36.8% at day 4) reached values analogous to E2F3 alone.

In summary, knockdown of E2F3 strongly inhibited cell proliferation in 6p22.3-amplified cells (−36.7%), whereas no such effect was observed for NM_017774 (+5%). This argues against a cooperative effect of E2F3 and NM_017774 on cell proliferation. Importantly, even after successful knockdown of E2F3 and NM_017774, the residual amount of mRNA left over in the amplified cell line exceeded the standard mRNA levels of non-amplified and non-siRNA–treated cells by a factor 5–10 (see Figure 4). Nevertheless, knockdown of E2F3 severely inhibited regular cell growth in 6p22.3-amplified cells. This emphasizes E2F3 as the relevant target gene of 6p22.3 amplification. Together with our recent observation that E2F3 expression is linked to rapid proliferation (Oeggerli *et al*., 2004), these data support an important role of E2F3 as a limiting factor for urothelial cell proliferation. It seems that 6p22.3 amplification conveys massive E2F3 overexpression in order to overcome a molecular bottleneck that prevents accelerated cell proliferation.

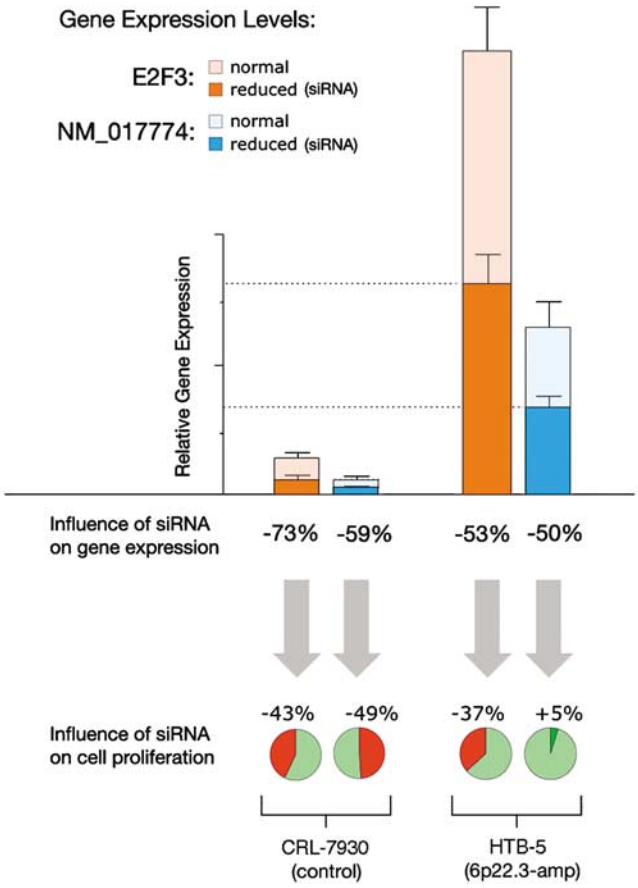

Figure 4 Effects of siRNA on E2F3- and NM_017774-specific mRNA levels are displayed for 6p22.3-amplified and non-amplified bladder cancer cell lines. In each case, the influence on cell proliferation was measured. Bars show that knockdown rates were comparable for both target genes, resulting in an always more than 50% reduction of individual mRNA levels. Pie charts at the bottom provide the complementary information whether reduced gene expression did affect cell growth or not. Values of reduced proliferation rates are noted above each pie chart (in percentage of the nonsense RNAi control samples). Importantly, in 6p22.3-amplified cell line HTB-5, only the reduction of E2F3 lead to decreased cell growth, whereas an equally strong reduction of NM_017774 had no negative effect on tumour cell proliferation.

Simultaneous knockdown levels of NM_017774 did not lead to reduced growth rates of 6p22.3-amplified cells. In non-amplified cells, however, reduced levels of NM_017774 induced a comparable proliferation reduction (−48.7%) as seen for E2F3 (−43.1%; see Figure 3). The growth reduction was even stronger if both E2F3 and NM_017774 were jointly silenced (−57.9%). The biological function of NM_017774 is unknown yet, but this finding adds additional evidence to the hypothesis (see above) that NM_017774 might be involved in regular cell growth. Conclusively, we found no evidence for NM_017774 to have a possible supportive effect on enhanced cellular proliferation when co-amplified and co-overexpressed alongside E2F3. Our results either suggest that NM_017774 is only accidentally

Table 2 Tumour categories with prevalent E2F3 amplification

Tumour categories	*Interpretable (n = 2296)*	*E2F3 amplified (n = 16)*
Breast cancer primary tumour	43	1
Breast cancer metastasis	181	1
Bladder cancer muscle invasive (pT2–4)	70	13
Bladder cancer non-invasive (pTaG2)	42	1

co-amplified because of its spatial neighbourhood to E2F3 (like other genes in the area) and does not have a functional role in 6p22.3 amplification, or that co-amplification of NM_017774 could be involved in another, not yet detected aspect of the disease, that is not linked to enhanced cellular proliferation.

Amplification of 6p22.3 has been reported exclusively in bladder cancer so far (Bruch *et al*., 2000; Feber *et al*., 2004; Hurst *et al*., 2004). The finding of a single case of breast cancer with 6p22.3 amplification in a multitumour TMA experiment, and a second breast cancer that was recently found to be E2F3 amplified in a DNA chip-based copy number analysis in our lab (C Ruiz, unpublished personal observation) (see Table 2) indicates that E2F3 amplification is not limited only to bladder cancer.

Conclusively, our data in conjunction with published evidence strongly indicate that amplification of E2F3 is a hallmark of one genetic pathway in invasive bladder cancer that is followed by approximately one-third of these tumours. These results prompt for a review of existing drug compound databases for potential E2F3 inhibitors.

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Curriculum vitae

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Personal data

Surname:	Oeggerli
First name:	Martin
Date and place of birth:	7th of March, 1974 in Riedholz (SO), Switzerland
Gender:	Male
Marital status:	unmarried
Nationality:	Swiss

Education

2002-2005 — **Ph.D. student at the University of Basel, Switzerland, Institute of Pathology; Dep. Molecular Pathology.**
Supervisor: Prof. Dr. Guido Sauter
Ph.D. thesis: "E2F3 is responsible for frequent amplification of 6p22.3 in human bladder cancer"
Supervisor of the Thesis: Prof. Dr. Guido Sauter
Referent and member of the Faculty: Prof. Dr. Alex N. Eberle
Co-referent of the exam committee: PD Dr. Ruth Chiquet-Ehrismann
Chairman of the exam committee: Prof. Dr. Tom Bickle

2000-2002 — **1 year diploma work at the Vertebrate Lab, University of Basel, Switzerland, Department of Vertebrate Zoology.**
Supervisors: **Prof. Dr. David G. Senn**
Received diploma in April 2002

1994-2000 — **Six years of studies in Biology I (classical Biology) at the University of Basel.**
Received certificate I in March 1996 and certificate II in March 1998

1989-1995 — **Matura school education in the Kantonsschule Solothurn, Switzerland.**
Received certification in January 1994

1985-1988 **Secondary school education at Wangen a/A, Bern, Switzerland**

1981-1984 **Primary school education at Riedholz, Solothurn, and Wangen a/A, Bern, Switzerland**

Oral presentations

2005 Evaluation of potential target genes inside the 6p22.3-amplicon in urinary bladder cancer

Poster presentations

2003 AACR, Washington D.C., United States

Publications

Year	Title	Authors	Journal
2004	E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer.	Oeggerli, M., Tomovska, S., Schraml, P., Calvano-Forte, D., Schafroth, S., Simon, R., Gasser, T., Mihatsch, M. J., and Sauter, G.	Oncogene 2004;23:5616-23
2005	Evaluation of Potential Target Genes of the 6p22.3-Amplicon in Urinary Bladder Cancer.	Oeggerli, M., Schraml, P., Novotny, H., Sauter, G., and Simon, R.	Verh. Dtsch. Ges. Path. 2005;89:000-000
2005	High-throughput tissue microarray analysis of CMYC amplificationin urinary bladder cancer	Zaharieva, B., Simon, R., Ruiz, C., Oeggerli, M., Mihatsch, M. J., Gasser, T., Sauter, G., Toncheva, D.	Int J Cancer; 2005: Dec 20;117(6):952-6
2006	E2F3 is the main target gene of the 6p22-amplicon with high specificity for human bladder cancer	Oeggerli, M., Schraml, P., Bloch, M., Ruiz, Ch., Novotny, H., Mirlacher, M., Sauter, G., and Simon, R.	Oncogene 2006: 25:6538-43

Attended courses at University of Basel during PhD

Nr.	*Attended Lecture*	*Assistant Professor*
2470	Cellular Signalling I	K. Ballmer-Hofer
8502	Cellular Signalling II	K. Ballmer-Hofer
2471	Experimentelle Krebsforschung I	M. Burger
5498	Experimentelle Krebsforschung II	M. Burger
4484	Cytoskeleton	C.-A. Schönenberger
8498	Cell Nucleus	B. Fahrenkrog
4383	Toxikologie	S. Krähenbühl
4400	Molekulare Mechanismen Toxikologie	S. Krähenbühl