

SHORT REPORTS

Aberrations of the Chk2 tumour suppressor in advanced urinary bladder cancer

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Checkpoint kinase 2 (Chk2) is a tumour suppressor and signal transducer in genome integrity checkpoints that coordinate cell-cycle progression with DNA repair or cell death in response to DNA damage. Defects of Chk2 occur in subsets of diverse sporadic malignancies and predispose to several types of hereditary carcinomas. However, the status of Chk2 in tumours of the urinary bladder remains unknown. Here, we report that among 58 advanced (grade T2–T4) human bladder carcinomas, immunohistochemical analysis revealed tumour-specific reduction or lack of Chk2 protein in 6 (10.3%) cases. Genetic analysis of the latter subset showed that a Chk2-negative carcinoma #668 harboured a truncating mutation 1100delC, in one Chk2 allele and loss of the corresponding second allele. The 1100delC mutation was also found in the germ line of this patient. Sequencing of TP53 in tumour #668 identified two missense mutations. Furthermore, the vast majority of the tumours showed ‘unscheduled’ activatory phosphorylation on Thr68 of Chk2 in the absence of any DNA-damaging treatment. Our results indicate that the otherwise dormant DNA damage signal transducer Chk2 is aberrantly and constitutively activated in invasive urinary bladder carcinomas, and that such likely proapoptotic checkpoint signalling can be disabled by inactivation of Chk2 and/or p53 tumour suppressors in subsets of these tumours.

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Cellular mechanisms of genome integrity maintenance are commonly deregulated in cancer and numerous components of the cell-cycle checkpoints, and DNA repair pathways qualify as either tumour suppressors or proto-oncogenes (Bartek and Lukas, 2001; Hoeijmakers, 2001; Khanna and Jackson, 2001). One of the emerging tumour suppressors implicated in responses to

DNA damage is Chk2 (also known as hCds1 or CHEK2), an evolutionarily conserved serine/threonine kinase homologous with yeast protein kinases Cds1/Rad53 (Bartek *et al.*, 2001). The 543-amino-acid-long human Chk2 protein encompasses the C-terminal kinase domain, the phosphoprotein-interaction forkhead-associated (FHA) domain between amino-acid residues 115 and 165 and the N-terminal regulatory domain rich in SQ/TQ motifs (Bartek *et al.*, 2001). Several of the SQ/TQ sites are phosphorylated in response to DNA damage by the upstream signalling kinases of the phosphatidylinositol 3 family, in particular, the ATM (Ataxia Telangiectasia-Mutated) kinase (Kastan and Lim, 2000; Abraham, 2001; Shiloh, 2003). Prominent among such N-terminal regulatory modifications of Chk2 is the phosphorylation of threonine 68 (Thr68-P), an ATM-mediated event and an early ‘marker’ of Chk2 activation primarily in response to genotoxic insults that cause DNA double-strand breaks (DSBs), such as ionizing radiation and various drugs (Ahn *et al.*, 2000, 2002; Matsuoka *et al.*, 2000; Melchionna *et al.*, 2000; Xu *et al.*, 2002). Once activated Chk2 transduces the DNA damage signal to numerous downstream targets such as p53, Cdc25A, Cdc25C, BRCA1, E2F1, Pml1 and probably other substrates whose biological functions become modified and contribute to cell-cycle arrest, enhanced DNA repair or apoptosis (Bartek and Lukas, 2003).

In mammalian cells, Chk2 is a stable protein expressed throughout the cell cycle, and also in quiescent cells and diverse postmitotic, differentiated tissues (Lukas *et al.*, 2001). This abundant and almost homogeneous expression in normal tissues, detectable as a nuclear signal in immunohistochemical staining with antibodies to Chk2 (Bartkova *et al.*, 2001; Lukas *et al.*, 2001), has facilitated search for aberrant downregulation or loss of this tumour suppressor protein in human tumour biopsy specimens. To date, such aberrantly low levels of Chk2 protein were found in subsets of human breast carcinomas (Sullivan *et al.*, 2002; Vahteristo *et al.*, 2002), testicular tumours (Bartkova *et al.*, 2001) and lymphomas (Tort *et al.*, 2002). Among the underlying causes of such low or undetectable Chk2 protein are somatic mutations or polymorphic variants of Chk2, which make the altered Chk2 proteins much more labile (subject to enhanced proteasome-mediated degradation), often combined with loss of heterozygosity, or a

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so far poorly understood downregulation of apparently wild-type Chk2 (Bartkova *et al.*, 2001; Lee *et al.*, 2001; Matsuoka *et al.*, 2001; Sullivan *et al.*, 2002; Tort *et al.*, 2002; Vahteristo *et al.*, 2002; Dong *et al.*, 2003). Genetic alterations of *Chk2* have been identified in a wide spectrum of human sporadic tumours including carcinomas of the breast (Sullivan *et al.*, 2002), lung (Haruki *et al.*, 2000; Matsuoka *et al.*, 2001), vulva (Reddy *et al.*, 2002), colon (Bell *et al.*, 1999), prostate (Dong *et al.*, 2003) and ovary (Miller *et al.*, 2002), sarcomas (Miller *et al.*, 2002) and lymphomas (Hofman *et al.*, 2001; Tavor *et al.*, 2001; Hangaishi *et al.*, 2002; Tort *et al.*, 2002). In addition, germline mutations of *Chk2* were identified in rare families with the Li-Fraumeni syndrome (Bell *et al.*, 1999; Vahteristo *et al.*, 2001) and some *Chk2* variants were found associated with familial carcinomas of the breast (Meijers-Heijboer *et al.*, 2002; Sodha *et al.*, 2002a, b; Vahteristo *et al.*, 2002; Schutte *et al.*, 2003) and prostate (Dong *et al.*, 2003; Seppälä *et al.*, 2003).

On the other hand, the role of Chk2 in tumorigenesis is far from understood, and the status of Chk2 in many types of human tumours remains unknown, including carcinoma of the urinary bladder, one of the most common human malignancies. Here, we report on an initial assessment of the Chk2 tumour suppressor protein in bladder cancer, through examination of its abundance, subcellular localization and activation status in a series of 58 human primary invasive carcinomas of the urinary bladder, combined with genetic analysis of *Chk2* in a subset of these tumours. Apart from the first evidence for aberrantly down-regulated protein expression or genetic loss-of-function of Chk2 in small subsets of these tumours, our study also revealed an unexpectedly large proportion of cases with tumour-specific, constitutive activation of Chk2 in this cohort of patients not previously exposed to any DNA-damaging treatment.

To assess the Chk2 protein directly *in situ* in normal versus tumour tissues, we employed a previously optimized immunoperoxidase staining protocol for formalin-fixed, paraffin-embedded archival specimens, and stained parallel sections with two mouse mono-

clonal antibodies recognizing distinct domains of human Chk2 (Bartkova *et al.*, 2001; Lukas *et al.*, 2001; Vahteristo *et al.*, 2002). Analysis of normal human urinary bladder tissues ($n = 5$) showed a homogeneous

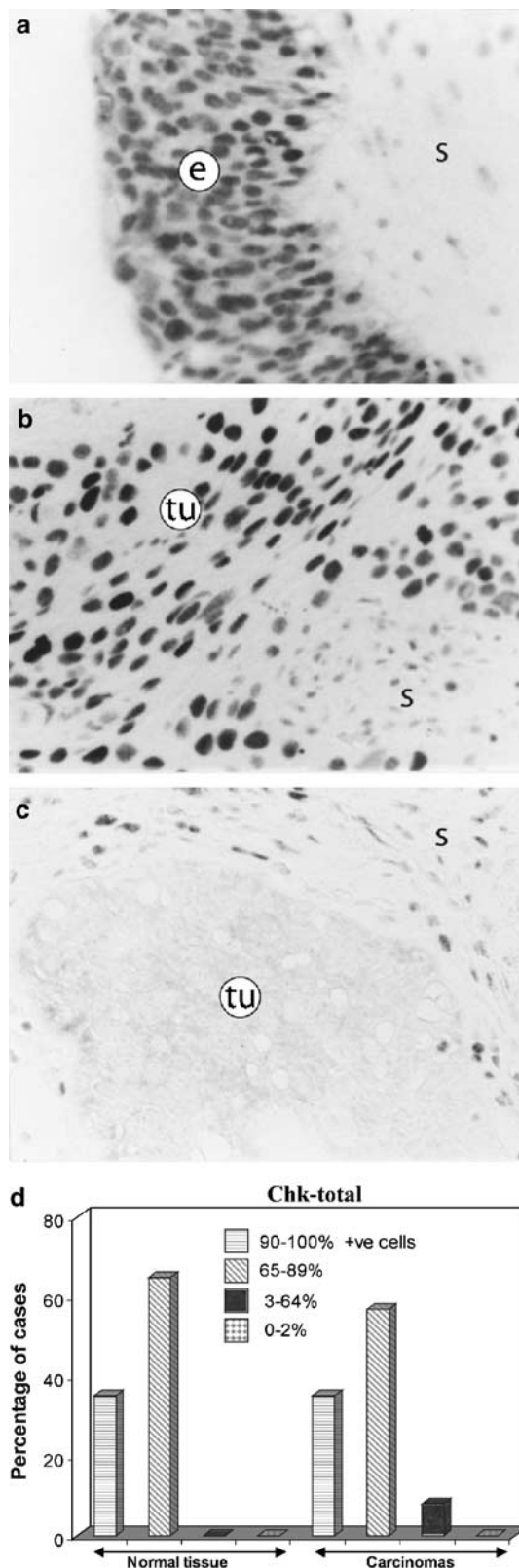


Figure 1 Immunohistochemical detection of Chk2 in normal human urinary bladder tissue and bladder carcinomas. (a) Immunoperoxidase staining (antibody DCS-270) of normal urinary bladder epithelium shows homogeneous nuclear positivity. (b and c) Two papillocarcinomas stained with antibody DCS-270 (S = stroma, Tu = tumour). Note the strong nuclear expression of Chk2 in both tumour and stromal cells in the carcinoma shown in (b), in contrast to very low-to-undetectable Chk2 staining in tumour cells of case #668 shown in (c). The lack of Chk2 staining in (c) is specific for cancer cells, as stromal cells present on the same section are positive. Controls including nonimmune mouse serum and the antibody DCS-270 preabsorbed with recombinant Chk2 were consistently negative, while parallel staining with another antibody against Chk2, DCS-273 (Lukas *et al.*, 2001) showed staining patterns similar to those observed with DCS-270. The tumours included in this study were from the Aarhus University tissue bank, of stages T2–T4 as classified according to the 1982 guidelines from UICC, and the study was approved by a local ethical committee and patients' informed consent. (d) Graphical summary of the immunohistochemical analysis of Chk2 in normal urinary bladder versus bladder carcinomas

nuclear staining pattern of Chk2 in virtually all cells of the transitional epithelium and nuclear staining of the majority of submucosal stromal cells (Figure 1a). Among the 58 primary bladder tumours of grade T2–T4 (57 uroepithelial carcinomas and one adenocarcinoma), resected surgically at the Aarhus University Hospital in the period between 1994 and 1998, only 18 (31%) specimens showed a homogeneous nuclear staining pattern of Chk2 comparable to normal uroepithelium (Figure 1b). In the remaining majority of tumour tissues Chk2 was also nuclear yet detectable in smaller and variable subsets of cells, and often with reduced intensity. In six cases (10.3%), the Chk2 downregulation was particularly apparent (Figure 1c and d). The reduction or lack of Chk2 staining was restricted to tumour cells, as the stromal elements surrounding the tumour nests preserved the normal Chk2 staining pattern (Figure 1c), thereby providing an internal control present on the same sections. We concluded from these results (summarized in Figure 1d) that the abundance of the Chk2 tumour suppressor protein is aberrantly downregulated or lost, to a widely variable degree, in subsets of human bladder carcinomas.

To examine whether the low abundance of the Chk2 protein seen in some bladder carcinomas in our series could reflect mutations of the *Chk2* gene, we analysed genomic DNA isolated from the six tumours with the most reduced Chk2 staining (those from the last two categories shown in the graph in Figure 1d) by denaturing gradient gel electrophoresis (DGGE). The entire coding sequence and all splice sites of the *Chk2* gene were scanned for mutations using the amplification primers listed in Table 1. As can be seen from Figure 2a, aberrantly migrating bands corresponding to amplified 5' segments of exon 10 (exon 10.1) were detected in samples from two separate areas of tumour #668, whereas *Chk2* DNA from the remaining five tumours

appeared to be wild type (Figure 2a and data not shown). Direct DNA sequence analysis of *Chk2* exon 10.1 from carcinoma #668 identified mutation 1100delC in one allele and loss of the wild-type allele (Figure 2b). Interestingly, the same 1100delC variant of *Chk2* has been reported to represent a low-penetrance breast cancer (Meijers-Heijboer *et al.*, 2002; Vahteristo *et al.*, 2002) or prostate cancer (Seppälä *et al.*, 2003) susceptibility allele in various human populations. Our data suggest that germline variants of *Chk2* may also be a predisposing factor in bladder cancer. Consistent with the presence of this *Chk2* sequence alteration already in the germ line, most probably in a heterozygous state, we detected both the wild-type and the 1100delC sequences during subsequent analysis of genomic DNA isolated from the peripheral blood cells of the patient #668 (data not shown). Collectively, these results imply that in the bladder carcinoma of the patient #668, the 1100delC allele was present already as a germ-line variant, whereas the loss of the second allele was a somatic event associated with the development of this tumour. This interpretation is entirely consistent with our immunohistochemical findings of extremely low-to-undetectable Chk2 protein in the cancer cells, as opposed to easily detectable Chk2 nuclear signal in the stromal cells of the tumour #668 (Figure 1c). Since the aberrant 1100delC protein is much more labile than the long-lived wild-type Chk2 (Lukas *et al.*, 2001, and our unpublished data), in the absence of the remaining wild-type allele, there is much lower overall protein level of Chk2 in the tumour cells than would be anticipated for a simple loss of 50% of the overall abundance due to loss of one of the two alleles. We propose that due to the extremely low abundance of the 1100delC protein and its deficiency in biological activity (reflecting truncation of this variant within the kinase domain), combined with the absence of wild-type Chk2, the cells of tumour #668 are functionally lacking any Chk2 kinase activity.

Table 1 Primers for DGGE analysis of the *CHEK2* gene

Exon	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')	Product length (bp)
1.1	TTGTTGGACACTTTCTTTTTGA	[50GC]GGGTAGGCTCCTCAGGTTC	337
1.2	[9GC]ACTCTATTCTATTCCTGAGGACC	[40GC]GAACAAAACGTGATACTATAACAACAA	195
2	[40GC]CCTCTGATGCATGCTTTTATA	[12GC]AGATACATGGGTATTCATTAC	229
3	[40GC]GGTCATTGTTTTAGATATTTT	[13GC]AATCCAGTAACCATAAGATAA	308
4	CCTCTGTGAATAAATTAATGAA	[40GC]ACCACCAATCACAAATGTATAG	242
5	[7GC]ATCACTATCTTTGTTTTCCC	[40GC]TGTTAATAAAAAGGTGATCAGC	220
6	[12GC]TTTTTAAATATTGGCAATTATTA	[40GC]TAAATCTAAGTATGAGTCATATAATAA	197
7	TACTTCCCTTTTTCTCCCC	[40GC]GAATGGAAACAGAAATTTTTAAAAA	171
8	GGAATGAACCCCTTGCCCTT	[40GC]ATTGAGGGAGTAATTCAACTAAAAAGA	235
9	ACTGCATGAATCTGAGGGTC	[40GC]CCACATACAGAATGCCAATTT	193
10.1	[40GC]TTATTCCTTTTGTACTGAAT	ATAACTCCTAAACTCCAGCA	218
10.2	GGTATAACCGTCTGTGGA	[40GC]AATCACCTCCTACCAGTCTGTG	136
11	[9GC]CCTTTCTCTCTACCAATATTA	[40GC]CAAAATCTTAACCCTTTCATA	218
12	[40GC]ACTGTGATTTGCCCAATTGTTG	GCTAGCAGGCACGTGCCCA	180
13	[40GC]GACACAGCTACTTATGTTTTAATT	[11GC]TTAGTGATCATCAGGAATACGA	190
14 ^a	[55GC]GAACATTTCTCCAccTTCC	ATTTCTTTCGTGTTCAAACCAC	202

[40GC] = CGCCCGCCGCGC0CCC GCGCCCGTCCC GCGCCCGCCCGCCCG.

[50GC] = CCGCGCCCGCCGCTCGCCCGCCGCGCCCGTCCC GCGCCCGCCCGCCCG.

[55GC] = CGCCCGCCGCGCCCGCCCGTCCC GCGCCCGCCGCGCCCGTCCC GCGCCCGCCCGCCCG. ^aLower-case characters represent nucleotides incorporated into the primer to modulate the melting characteristics of the amplicon

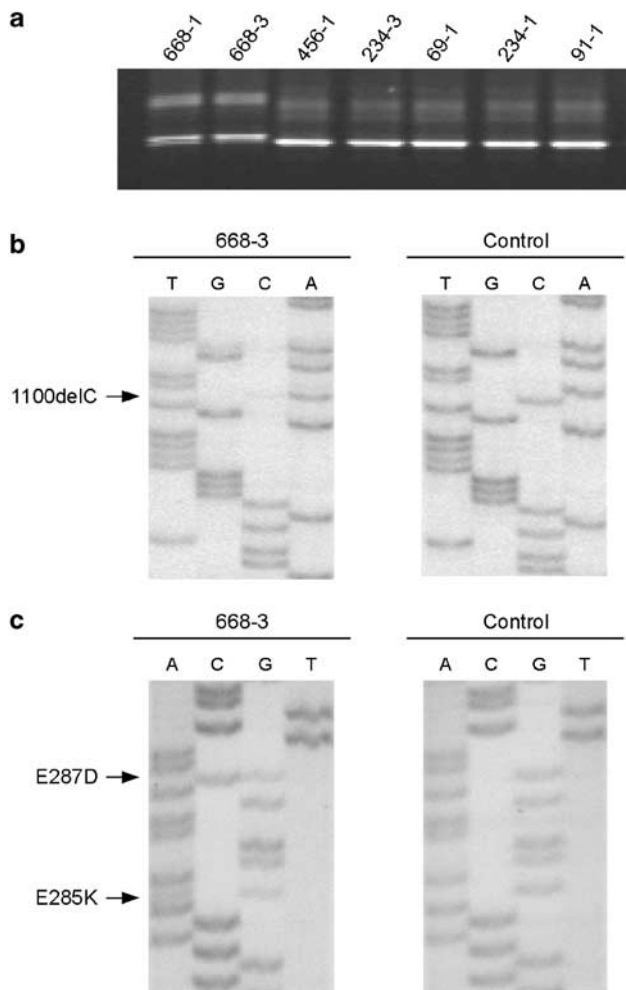


Figure 2 Mutational analysis of the *CHEK2* and *TP53* genes in a subset of bladder carcinoma cases, and in case #668, respectively. DNA was extracted from tissue using the Puregene extraction kit (Gentra Systems) and following the manufacturer's instructions. Primer sequences for DGGE analysis of *Chk2* are listed in Table 1. For analysis of exons 10–14, a ~9.2 kb region encompassing these exons was first amplified by long-range PCR, using previously described primers and conditions (Sodha *et al.*, 2002a,b). The products of these reactions were used as a template in a second round of PCR using exon-specific primers. PCR products were analysed in a 0% denaturant/6% polyacrylamide–80% denaturant/12% polyacrylamide double-gradient gel. Primer sequences and conditions for DGGE analysis of the *TP53* gene were as described (Guldberg *et al.*, 1997). (a) DGGE analysis of a region spanning the 5'-end of *Chk2* exon 10, showing aberrant bands in MOB 668-1 and MOB 668-3 (two biopsy samples originally taken from different parts of tumour #668). (b) Direct sequence analysis of *Chk2* exon 10.1, showing the 1100delC mutation and loss of the wild-type *Chk2* allele in MOB 668-3. (c) Sequence analysis of *TP53* exon 8, showing the E285K and E287D mutations in MOB 668-3

Such a scenario is entirely consistent with loss-of-function alterations characteristic of other tumour suppressors.

The role of Chk2 as a kinase that regulates the function of p53 in response to some genotoxic stimuli (Chehab *et al.*, 2000; Hirao *et al.*, 2000, 2002; Shieh *et al.*, 2000; Falck *et al.*, 2001; Takai *et al.*, 2002; Craig *et al.*,

2003; Lukas *et al.*, 2003), and the original finding of Chk2 and p53 mutations in mutually exclusive subsets of families suffering from the tumour-prone Li–Fraumeni syndrome (Bell *et al.*, 1999) led us to investigate the status of p53 in the tumour #668 that lacks functional Chk2. Immunohistochemical staining showed a highly abundant nuclear p53 protein indicative of mutant p53 (data not shown), and combined DGGE and sequence analysis of the *TP53* gene (Guldberg *et al.*, 1997) revealed two missense mutations, E285K and E287D, both in exon 8 (Figure 2c). Regardless of whether these two mutations of *TP53* reside in the same or distinct alleles of the gene, these data demonstrate that carcinoma #668 harbours concomitant defects of both Chk2 and p53. Such a scenario is reminiscent of concomitant mutations of *TP53* and *Chk2* genes reported for some other types of sporadic tumours (Falck *et al.*, 2001; Reddy *et al.*, 2002; Sullivan *et al.*, 2002), or positive association of mutations in *ATM* (the upstream regulator of Chk2) with inactivation of the *ARF–TP53* pathway (Grønbaek *et al.*, 2002). Coexistence of aberrations in two components of the same signalling pathway in the same tumour is conceptually interesting likely reflecting the diverse partly redundant modes of p53 activation multiple substrates of Chk2 in response to various genotoxic stresses (see, Bartek and Lukas, 2003, for a more detailed discussion of this topic). Given the occurrence of Chk2 and p53 aberrations either alone or concomitantly, it appears that even after loss of function of either Chk2 or p53, the engagement of the other, still functional protein in the remaining operational genome integrity pathways provides sufficient pressure to select for cells that sustained its inactivating alteration during subsequent tumour progression.

The early 'marker' of Chk2 activation, the ATM-mediated phosphorylation of Chk2 on threonine 68, can be detected with a carefully characterized phosphospecific antibody in cultured cells (DiTullio *et al.*, 2002; Lukas *et al.*, 2003). We have also recently validated this approach for monitoring activated Chk2 by staining with antibody to Thr68-P directly on archival tissue specimens (DiTullio *et al.*, 2002). To better assess the functional state of Chk2 in the urinary bladder tissues and tumours, we employed this strategy, and examples of characteristic staining patterns are shown in Figure 3a–e. Overall, there was a striking difference between the normal urothelium samples that showed no indication of Chk2 activation despite the abundant expression of Chk2 (Figure 3a and b), as opposed to the vast majority of carcinomas in which Chk2 was phosphorylated on Thr68 in variable subsets of cells (Figure 3c–f). Importantly, this activation of Chk2 was restricted to tumour cells (Figure 3d and e), and it was independent of proliferation rate, as there was no correlation of Chk2-Thr68 phosphorylation and the fraction of cells positively stained with an antibody against the proliferation marker Ki67 used on parallel tissue sections (data not shown). Most significantly, the frequent and often pronounced activation of Chk2 in this series of bladder carcinomas (Figure 3f) was constitutive and

'unscheduled', since none of the patients were treated by any DNA-damaging treatments such as radiation or chemotherapy before surgery.

The results of the present study contribute to our understanding of the role Chk2 may play in tumorigenesis in at least two ways. First, we show that analogous to some other types of cancer, Chk2 is downregulated or lost in a small but detectable subset of urinary bladder carcinomas. Tumour-specific downregulation of Chk2 protein abundance without detectable sequence alteration, analogous to several cases in our series of bladder carcinomas, has been reported for subsets of breast cancer and lymphoid tumours (Sullivan *et al.*, 2002; Tort *et al.*, 2002). Promoter methylation has been excluded as the underlying cause of such decreased Chk2 levels (Sullivan *et al.*, 2002; Tort *et al.*, 2002), and the mechanism of this aberration remains unclear. The 1100delC variant of *Chk2* found in case #668 is known to predispose to familial breast cancer with only low penetrance and without altering the age of onset of breast cancer, in contrast to *BRCA1* or *BRCA2* aberrations that have greater impact in breast cancer pathogenesis (see, Meijers-Heijboer *et al.*, 2002; Vahteristo *et al.*, 2002; Bartek and Lukas, 2003, for discussion). The behaviour of Chk2 as only a 'mild' tumour suppressor in human cancer is also consistent with the fact that deletion of *Chk2* neither affects viability and development of the *Chk2*-deficient mice nor does it significantly increase their frequency of spontaneous tumours until possibly late in their lives (Hirao *et al.*, 2002b; Takai *et al.*, 2002). On the other hand, the tumour incidence in *Chk2*^{-/-} mice is increased compared to wild-type animals when they are treated with DNA-damaging carcinogens. This, together with the fact that human Chk2 appears to become activated only in response to DSBs, suggests that Chk2 may represent a 'conditional tumour suppressor' whose loss can contribute to tumorigenesis mainly when the target cell is challenged by DNA damage.

The other aspect of emerging Chk2 involvement in tumorigenesis, highlighted by our study, is the high proportion of untreated urinary bladder carcinomas in

which Chk2 seems activated (as judged from its activatory phosphorylation on Thr68) 'constitutively', under conditions with no exposure to external DNA damage. This intriguing phenomenon was first reported for untreated breast and lung cancer (DiTullio *et al.*, 2002); however, the subsets of those tumours with Thr68-phosphorylated Chk2 were smaller compared with the bladder tumours examined here. It is unclear what causes this pronounced yet variable degree of Chk2 activation, but it is evident from our data that it

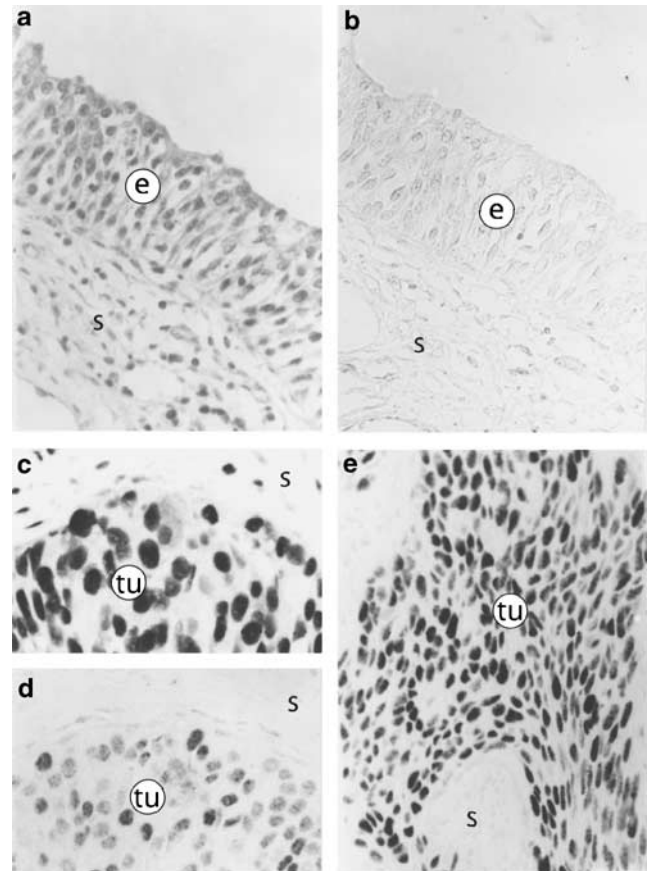
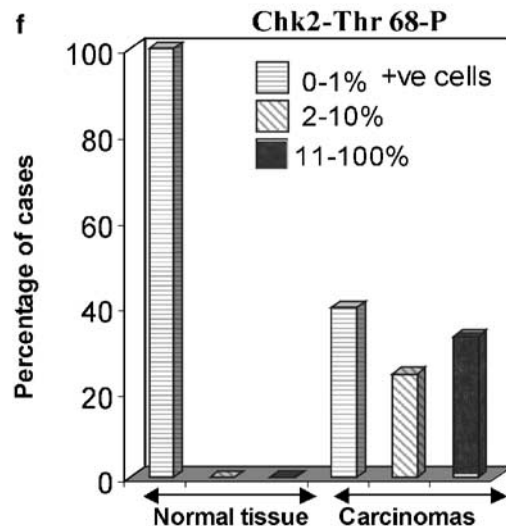


Figure 3 Immunohistochemical detection of Chk2 and its activated, Thr-68-phosphorylated form in human urinary bladder carcinomas. (a and b) Immunoperoxidase staining of parallel sections of normal human urinary bladder epithelium for the Chk2 protein (antibody DCS-270; Lukas *et al.*, 2001) and the activated form of Chk2 phosphorylated on Thr68 (rabbit phosphospecific antibody from Cell Signaling, Lot 1; diluted 1:500), respectively (S = stroma, E = epithelium). Note abundant Chk2 expression (a) and the lack of Chk2-Thr68 phosphorylation (b). (c and d) Serial sections of a urinary bladder papillocarcinoma stained with antibody DCS-270 against Chk2 and the phosphospecific antibody against Thr68-phosphorylated Chk2, respectively (S = stroma, Tu = tumour). Note the strong nuclear expression of Chk2 in both tumour and stromal cells (c), and a variable degree of Chk2-Thr68 phosphorylation in carcinoma cells (d). (e) Almost homogeneous and strong staining for Thr68-phosphorylated Chk2 in tumour cells of another carcinoma, detected with the phosphospecific antibody (note the absence of Chk2 phosphorylation in stromal cells; S = stroma, Tu = tumour). (f) Graphical summary of the immunohistochemical analysis of Thr68-phosphorylated Chk2 in normal urinary bladder tissue versus bladder carcinomas



occurs selectively in the tumour cells, and we also noticed that the carcinomas with the highest proportion of Thr68-P-positive cells are at more advanced stages of progression. As Chk2 is normally activated by the ATM kinase, it is plausible to speculate that also under these pathological conditions, ATM itself could be activated aberrantly, and this would lead to phosphorylation of Chk2 and possibly other targets of ATM such as p53. Among the plausible cancer-associated abnormalities that could underlie this phenomenon are oncogenic activation or abnormal telomere maintenance, both scenarios likely mimicking 'constitutive DNA damage' of the type known to evoke Chk2 activation (Bartek *et al.*, 2001; Bartek and Lukas, 2003; d'Adda di Fagagna *et al.*, 2003; Takai *et al.*, 2003). Regardless of the stimuli that lead to such unscheduled activation of Chk2, this state would normally signal to downstream effectors of the checkpoint pathways such as Cdc25, Pml1, E2F1 or

p53, and eventually lead to cell-cycle arrest or apoptosis. We believe that this is an attractive concept, complementary to the 'spontaneous' tumour-specific activation of the ARF tumour suppressor, recently reported to occur in mouse models (Zindy *et al.*, 2003). We propose that such environments with activated tumour suppressor mechanisms may provide 'evolutionary' pressure to select for variant cancer cells with defects in cell-cycle machinery and mutations resulting in loss of pro-apoptotic genes or aberrant activation of survival factors, all hallmarks of advanced tumorigenesis.

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