In search of genes responsible for the development of renal cell carcinoma

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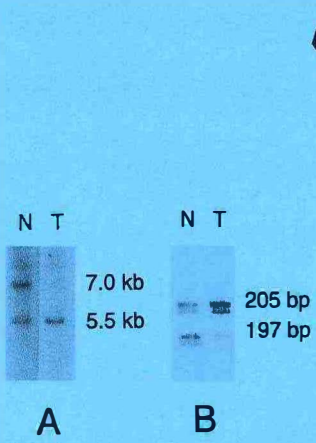

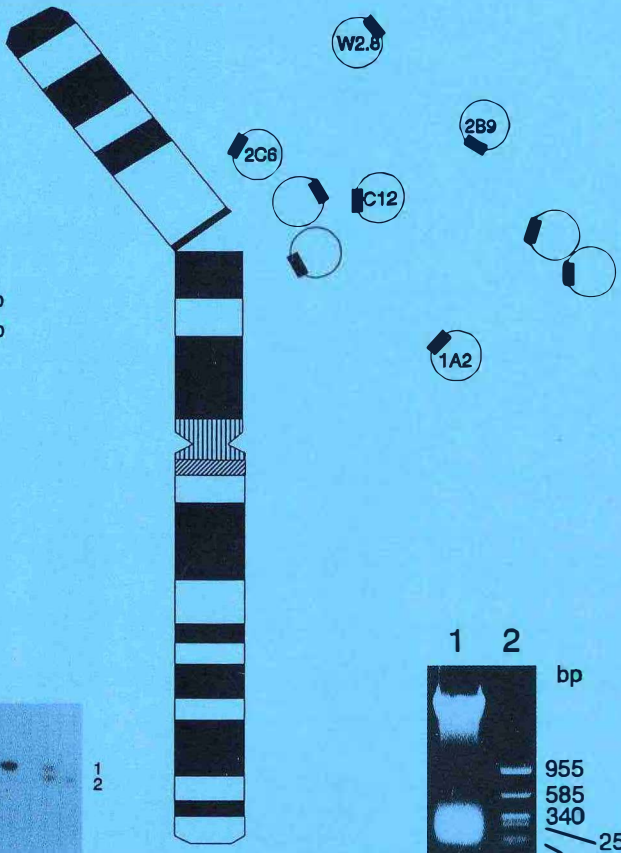

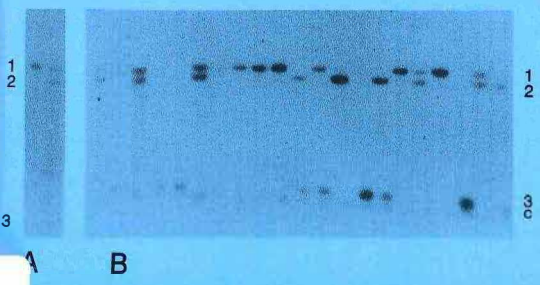

A. H. van der Hout

IN SEARCH OF GENES RESPONSIBLE FOR THE DEVELOPMENT OF RENAL CELL CARCINOMA

STELLINGEN

1.

Fysische en genetische genoomkarteringsstudies verliezen veel van hun waarde als geen algemeen geaccepteerde referentie-markers in het onderzoek betrokken worden.

2.

Het is onwaarschijnlijk dat er bij fluorescentie-in situ hybridisatie een verband bestaat tussen de grootte van de chromosoomband waarop een DNA-merker gelokaliseerd wordt en de grootte en helderheid van het hybridisatiesignaal. (Takahashi et al., Genomics 13 (1992) 1047-1055)

3.

Uit de resultaten van restrictie-fragment-lengte-polymorfisme analyse in adenocarcinomen van de long door Yokota et al. (Cancer Research 52 (1992) 873-877) zijn geen 2 maar 3 verschillende deletiegebieden op de korte arm van chromosoom 3 af te leiden.

4.

Het is niet te verwachten dat de specifieke combinatie van cytogenetische afwijkingen: -Y,+7,+7,+17 ooit in niertumoren van vrouwelijke patiënten gevonden zal worden (Meloni et al., Journal of Urology 148 (1992) 253-265).

5.

Het moeilijkste punt in de speurtocht naar tumorsuppressorgenen is het aantonen dat het gezochte gen gevonden is.

6.

Wanneer in een deel van een gen eenmaal enkele mutaties gerapporteerd zijn, houdt een dergelijke mutatie-hotspot zichzelf in de literatuur in stand.

Stellingen
behorende bij het proefschrift van
A. H. van der Hout

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Groningen, 12 mei 1993

Rijksuniversiteit Groningen

IN SEARCH OF GENES RESPONSIBLE FOR THE DEVELOPMENT OF RENAL CELL CARCINOMA

Proefschrift

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geboren op 27 april 1959
te Groningen

Promotor: Prof. Dr C.H.C.M. Buys

Contents

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CHAPTER 1

General introduction

1.1 Sporadic renal cell carcinoma

Renal cell carcinoma (RCC), the most common malignancy of the kidney in adults, affects 5 to 10 per 100,000 persons and thereby accounts for 2%-3% of all adult cancers. The median age at diagnosis for sporadic RCC is 60 years, but RCC can also affect children and young adults (de Jong et al., 1986, Tomlinson et al., 1991, Dal Cin et al, 1991). Men are affected twice as often as women (Cronin et al., 1991). Cigarette smoking seems to be a risk factor (Bennington and Laubscher, 1968, La Vecchia et al., 1990), which may contribute to this sex difference in occurrence. Most RCC cases are sporadic but rare familial cases occur (Li et al., 1982). RCC is also one of the tumours for which patients with the hereditary Von Hippel Lindau syndrome (VHL) are at risk.

1.1.2 Chromosome analysis

The first chromosome analysis of an RCC tumour was published by Pathak et al. (1982). From this study and many more that followed (reviewed in Walter et al., 1989, Meloni et al., 1992) it appeared that the short arm of chromosome 3 -3p- is the chromosome arm most often involved in abnormalities. A deletion in the region 3p13-pter has been found in up to 95% of cases studied (Kovacs and Frisch, 1989). Other chromosomes frequently involved in numerical and structural abnormalities are chromosomes 5, 7, and 14 (Kovacs and Frisch, 1989; Presti et al., 1991). Cells with trisomy 7 as their only karyotypic abnormality are often found. However, this abnormality has also been found in normal kidney tissue of RCC patients (Kovacs and Brusa, 1989; Elfving et al., 1990) and in kidneys with

a non-neoplastic pathology (Casalone et al., 1992; Emanuel et al., 1992). Dal Cin et al. (1992) found tumour infiltrating lymphocytes with trisomy 7 or trisomy 10 in RCC tumours and surrounding kidney tissue. Therefore, trisomy 7 is probably not an abnormality specific for RCC tumour cells. The significance of the presence of lymphocytes with these trisomies in and near RCC tumours remains unknown. The cytogenetic analysis of sporadic RCC will be dealt with in more detail in Chapter 4, DNA analysis will be discussed in Chapter 2.

1.2 Familial renal cell carcinoma

1.2.1 Pure familial renal cell carcinoma

The term 'pure familial RCC' is used to make the distinction with RCC in VHL, a syndrome in which apart from RCC also other tumours occur. Thirty-two family aggregates with pure RCC have been reported in the literature (cited in Li et al., 1982; Erlandsson et al., 1988). For patients in these families the median age at diagnosis was a good 10 years younger than for sporadic cases and both bilateral occurrence of tumours and occurrence of multifocal tumours were much more frequent. In sporadic RCC 1% of cases is bilateral, whereas Li et al. (1982) report bilaterality in 15 out of 42 cases (36%) with familial RCC.

In most families with pure familial RCC no consistent chromosome abnormalities have been found (Li et al., 1982; Pathak and Goodacre, 1986). In a family reported by Cohen et al. (1979), however, a balanced constitutional translocation (3;8)(p21;q24) was found. The breakpoints have been defined more precisely by Wang and Perkins (1984) as 3p14.2 and 8q24.1. In 3 consecutive generations of this family 10 members had been affected with RCC. Whereas in all analyzable cases (n=5) the patients had inherited the translocation, RCC did not occur in any family member who was not a carrier of the t(3;8). The median age at diagnosis for the patients in this family was relatively young (45 years), 6 out of 10 cases were bilateral and 8 out of 10 multifocal. One RCC of a member of this family has been studied cytogenetically (Decker et al., 1992). Apart from the observation of a complex translocation involving chromosomes 13 and 16, and loss of chromosomes 8, 13, and 14 it appeared that the der(8) chromosome was

lost in the tumour, whereas the normal 3 and the der(3) were retained. Thus, from chromosome 3 the segment 3p14.2-pter got deleted.

Kovacs et al. (1989) described a family with a balanced constitutional translocation (3;6) with breakpoints between 3p13 and 3p14 and at 6q25.1 in three family members in three consecutive generations. The oldest translocation carrier was diagnosed at 53 years of age with multiple bilateral RCCs. The other two carriers were too young (27 and 3 years) to have developed RCC already. Therefore, in this family it is too early to establish whether RCC segregates with the translocation. Cytogenetic studies revealed that the 3p13-pter segment was lost in the tumours of the oldest translocation carrier, similar to the situation in the t(3;8) family.

Pathak et al. (1982) described that in tumour cells of a patient with familial RCC, among other abnormalities, a reciprocal translocation (3;11) occurred with a breakpoint in 3p13. There was no obvious deletion of 3p material. The translocation was not found in the patient's constitutional cells, which makes it unlikely that the translocation has something to do with the heritable nature of tumour development in this case. In summary, the very limited evidence available might point at a 'familial RCC gene' which should then be located near the familial 3p breakpoints, i.e. most likely in band 3p14. This chromosome region will be discussed in Chapter 3.

1.2.2 Renal cell carcinoma in Von Hippel-Lindau syndrome

RCC is one of the clinical manifestations of the Von Hippel-Lindau syndrome (VHL). VHL is a hereditary disorder, characterized by an inherited predisposition to develop neoplastic lesions in certain organs. Apart from RCC, which occurs in 30-40% of VHL patients the most frequent lesions are retinal angiomas, cerebellar and spinal haemangioblastomas, phaeochromocytomas and renal, pancreatic, and epididymal cysts.

Inheritance of VHL is autosomal dominant. The incidence is about one in 40,000 persons. The risk to develop RCC for VHL patients rises to 70% at the age of 60 years. As in pure familial RCC, RCC in VHL is mostly bilateral and multifocal. Also the mean age at diagnosis (about 45 years) is similar for these

two types of familial RCC (Maher and Yates, 1991). The histopathological appearance of all forms of inherited RCC are similar to those of sporadic RCC. The presence of multiple renal cysts, however, may be characteristic for VHL. It has been proposed that these cysts may be considered as precancerous precursors of RCC in VHL (Solomon and Schwartz, 1988).

No specific chromosome abnormalities could be detected in lymphocytes of patients with VHL (Go et al., 1984; Neumann et al., 1988). Cytogenetic studies on 46 RCC tumours from 12 VHL patients were summarized by Kovacs et al. (1991). In all cases loss of the 3p13-pter region was found. It was noticed that loss of chromosome 14 was much less frequent in RCC in VHL than in sporadic RCC (10% vs. 50%). Bergerheim et al. (1990) have reported bilateral RCCs from a VHL patient with a del(4)(p14) and apparently normal copies of chromosome 3 in both tumours. Kiechle-Schwarz et al. (1989) did a chromosome analysis of 3 phaeochromocytomas from VHL patients. The only tumour with specific chromosome abnormalities showed 4 different clones of which 3 showed rearrangements resulting in trisomy of at least the 3p21-p26 segment. In 3 haemangioblastomas and 5 phaeochromocytomas from VHL patients Jordan et al. (1989) found no microscopically apparent clonal chromosome abnormalities. Tory et al. (1989), however, who investigated loss of heterozygosity (LOH) on 3p in tumours of 7 VHL patients, detected LOH in all RCCs (n=11), one phaeochromocytoma, one cerebellar haemangioblastoma, and two spinal haemangioblastomas. Retention of heterozygosity at all informative loci was only observed in two other spinal haemangioblastomas, that according to the authors may well have contained only a small percentage of tumour cells. In all cases with LOH the deletion involved at least the region distal to D3F15S2. For one patient four different RCCs could be studied. In all tumours loss of the same 3p alleles was found. These appeared to have been inherited from the unaffected father. Also in an RCC and a phaeochromocytoma from two other patients it could be determined that the 3p alleles lost in the tumours had been transmitted by the unaffected parent (in one case the father, in the other case the mother). To investigate whether 3p allele loss in VHL is specific, 9 tumours of three patients were examined for LOH at 12 loci on 9 chromosomes different from chromosome 3. No consistent losses were found. This indicates that,

consistent with the results of cytogenetic analysis, LOH on 3p is a fairly specific event in VHL.

Genetic linkage has been established between VHL and the RAF1 locus at 3p25 (Seizinger et al., 1988, Vance et al., 1990). More extensive linkage analysis has localized the VHL gene in the interval between RAF1 and D3S18 (3p26) (Hosoe et al, 1990).

1.3 The tumour suppressor gene model

A general model has been proposed in which a tumour cell is thought to develop as the result of loss or inactivation of both alleles of a proliferation suppressing gene. This model was originally developed for retinoblastoma (Knudson, 1971). From an analysis of age-incidence curves for familial and sporadic retinoblastoma the suggestion arose that the development of a sporadic cancer required two mutations and the development of a familial case only one. Comings (1973) proposed that the two mutations occurred in the two alleles of one and the same gene. In familial cases susceptibility to tumour development would be inherited by a germ-line mutation and a tumour would develop when the normal balancing wild-type allele was lost or inactivated by a somatic change, thereby unmasking the recessive mutant allele. In sporadic cases both alleles have to become inactivated in the same cell by two somatic events. This model explains why in contrast to sporadic retinoblastoma the familial form is often bilateral and multifocal and occurs in children who are on average younger than those affected by sporadic retinoblastoma.

A clue to where to look for the affected gene in retinoblastoma has come from cytogenetic studies. In blood lymphocytes from some patients a microscopic deletion of band 13q14 was observed (Francke, 1978). An analysis of combinations of DNA from white blood cells and from tumour tissue with DNA probes recognizing restriction fragment length polymorphisms (RFLPs) showed loss of chromosome 13 sequences in the tumours supporting the idea of unmasking a recessive mutation in the other allele (Cavenee et al., 1983). Eventually a gene was cloned from

13q14 which appeared to be mutated in retinoblastoma patients (Friend et al., 1986).

1.3.1 Tumour suppressor genes in renal cell carcinoma

There are a number of similarities between RCC and retinoblastoma. Although RCC is a cancer with mostly a late age of onset whereas retinoblastoma is an embryonic and childhood cancer, both occur in a familial and a sporadic form. In both tumour types, familial cases are more often bilateral and multifocal and develop at a younger age. Epidemiological studies have indicated that the retinoblastoma model can be applied to RCC: there are two rate-limiting genetic events in the development of the sporadic form whereas for familial RCC only one (somatic) event is rate-limiting (Erlandsson et al., 1988). Study of age-incidence curves for RCC and cerebellar haemangioblastoma in VHL versus sporadic cases led to a similar conclusion (Maher et al., 1990). Following the retinoblastoma paradigm germline abnormalities in familial cases should indicate the location of an 'RCC gene'. The constitutional t(3;8) found in the family described by Cohen et al. (1979) (see section 1.2.1) could represent such an abnormality. The frequent rearrangements of 3p found in cytogenetic studies of sporadic RCC also indicate a role for this chromosome arm. Another line of evidence points to a role for chromosome 3p in the development of RCC. The introduction by microcell-mediated transfer of a chromosome der(X)t(X;3)(Xq26;3p12) or of a chromosome consisting of 3pter-q13 and unidentified chromosome segments into an RCC cell line resulted in suppression of tumorigenicity or modulation of the tumour growth rate after injection into nude mice. The introduction of chromosomes 11 or X had no such effect (Shimizu et al., 1990). Killary et al. (1992) did similar experiments. They showed that the introduction of chromosome 3 in mouse fibrosarcoma cells suppressed tumorigenicity, whereas the introduction of chromosomes 2 or X failed to exert any tumour suppressive effect. A hybrid containing a fragment smaller than 2 Mb encompassing the region 3p21 near the interface with 3p22 had the same effect as a whole chromosome 3, localizing a tumour suppressor locus in this region.

This made it reasonable to focus the search for a gene of which the loss of function would be responsible for the development of RCC on the short arm of chromosome 3. Since no clues exist on what the gene product of this gene might be, cloning strategies had to be aimed at the exact location of the gene (positional cloning; reviewed by Collins, 1992). By studying loss of heterozygosity with polymorphic DNA probes rearrangements may be found that are undetectable by cytogenetic analysis and may help in defining a smallest region of common deletions in which the gene should reside.

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Chapter 2

The role of the chromosomal region 3p21-p24 in sporadic renal cell carcinoma

2.1 Overview

The involvement of 3p in chromosomal rearrangements in sporadic RCC as detected by microscopic analysis actuated molecular genetic studies of this chromosome arm. An analysis of 3p deletions could be readily carried out using polymorphic DNA markers specific for 3p in a comparative Southern blot analysis of matched pairs of RCC tumour and normal kidney tissue. The advantage of this method over conventional karyotyping is that there is no need for culturing tumour cells or for the direct preparation of chromosome spreads from solid tumour material (see section 2.2).

We studied 58 tumour/normal kidney tissue combinations with 10 markers recognizing 15 RFLPs on 3p and with 3 markers recognizing 3 RFLPs on 3q (see sections 2.3 and 4.2). All but one of the cases were informative for at least one locus on 3p. Loss of heterozygosity (LOH) on 3p was found in 29 of 57 informative cases (50%). On 3q LOH was found in only 5 of 39 informative cases (12%). This justifies the search for a common deletion to be aimed primarily at 3p. Moreover, it may be concluded that loss of a complete chromosome 3 homologue does not seem to be a common mechanism underlying allelic loss in sporadic RCC. At first instance the region bordered by THRB in 3p24 and D3S2 in 3p21 was found to be the smallest region of overlap for the deletions in these tumours (c.f. section 2.2). Extension of the study with the marker pEFD145.1 defining the locus D3S32 led to a further reduction of this region (Fig.1). With in situ hybridization D3S32 has been assigned to 3p21.2-21.3 (Yamakawa et al., 1991a). Double colour fluorescent in situ hybridization as well as somatic cell hybrid analysis placed D3S32 distal to D3S2 (P.H. Rabbitts, A.C. Heppell-Parton, personal communication; Drabkin et al., 1989,

Brauch et al., 1990, Gemmill et al., 1991). The definition of the interval between D3S32 and THRB as the smallest overlap of deletions in our series of sporadic RCC made us conclude that in view of the non-overlapping regions where candidate genes should be located (c.f. sections 1.1.1 and 1.1.2), different genes are involved in sporadic RCC, pure familial RCC, and VHL.

Ogawa et al. (1991) described one case with retention of heterozygosity at D3S4 (in 3p13-14; Heppell-Parton et al., 1992) and at D3S32, but with LOH at THRB. Their results confirm D3S32 as the proximal border of a possible common deletion region. Anglard et al. (1991) mapped deletions in 60 cases of sporadic RCC using mostly the same markers as we did. They found LOH for at least one marker in 51 of 58 informative cases. In 5 cases a partial 3p deletion was detected which made it possible to define a common deletion region bordered by D3S2 and D3S22. The latter locus is found in the telomeric part of 3p, distal of RAF1 which is in 3p25 (Tory et al., 1992) (Fig.2). Thus, our common deletion region is contained within the one found by Anglard et al. (1991). The estimated physical length of the interval THRB-D3S32 defining our common deletion region is 34 Mb, taking chromosome 3 as 7% of the genome (i.e. 210 Mb) (Harris et al., 1986). This is an interval much too large to start looking for a specific gene.

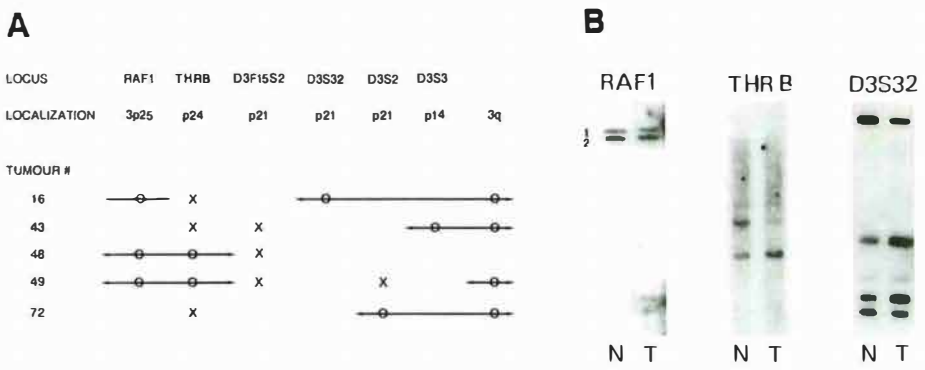

Fig.1. (A) Schematic representation of partial deletions of 3p in RCC. Arrows indicate the minimal regions of chromosome 3 that are not deleted in the tumours. X: loss of heterozygosity; O: retention of heterozygosity; no entry: locus not informative.
(B) Southern analysis of case 16, showing loss of heterozygosity at the THRB locus and retention of heterozygosity at RAF1 and D3S32. The longest allele of each RFLP is marked 1, the shortest allele 2.

Yamakawa et al. (1991a) have also performed a detailed deletion mapping of 3p in sporadic RCC. Using 24 polymorphic 3p markers, all localized by in situ hybridization on prometaphase chromosomes (Yamakawa et al., 1991b, Takahashi et al., 1992) they found 18 sporadic RCCs with partial or interstitial deletions of 3p. From 12 of these cases a commonly deleted region bordered by the markers D3S660 (3p21.2-p21.3) and D3S685 (3p21.3-p22.3) could be deduced. They also found three tumours which show retention of heterozygosity in the 3p21 region but allelic losses with more centromeric markers. The distal border of this 'proximal deletion region' was found to be D3S936 in 3p21.1. Moreover, they described three tumours which showed simultaneous LOH both proximal to D3S936 and in the 3p21.3 region, with retention of heterozygosity at loci located in between. The proximal deletions will be discussed in chapter 3. In one of the 'double deletion cases' (their tumour 38) retention of heterozygosity is found at a 3p21.3 locus, D3S686, located distal to D3S660. Combining the 12 single deletion cases and the 3 double deletion cases a distal commonly deleted region was found in 3p21.3 bordered by D3S686 on the proximal and D3S685 on the distal side. Thus, these results substantially shorten our common deletion region on the distal side. Since D3S686 was localized distal to D3S32 by in situ hybridisation our deletion is also somewhat shortened on the proximal side by the results of Yamakawa et al. (1991a).

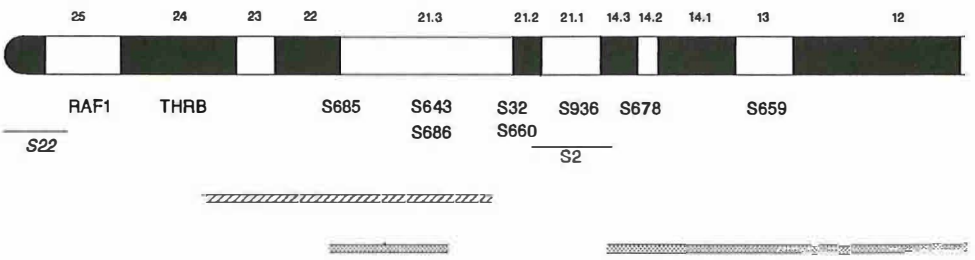

Fig.2 Common deletion regions in sporadic RCC as defined by us ▨ *and by Yamakawa et al. (1991)* ▩. *Localization of loci by in situ hybridization (Bonner et al., 1984, Albertson et al., 1989, Takahashi et al., 1992, Heppell-Parton et al., 1992), except D3S22 which was localized by linkage analysis (Tory et al., 1992). The prefix D3 was omitted from all anonymous loci.*

Also in lung cancer (Naylor et al.,1987, Kok et al., 1987, Rabbitts et al., 1989, Brauch et al., 1990, Yokoyama et al., 1992) and cervical cancer (Yokota et al., 1989) 3p is the chromosomal arm on which allelic losses are most frequently observed. From deletion mapping studies in lung cancer, using the same markers as Yamakawa et al. (1991a) did in their deletion mapping of RCC a rather complex picture emerges with even 5 different common deletion regions (Hibi et al., 1992, Yokoyama et al., 1992). Two of these common deletion regions fall within the distal deletion region in RCC as defined by Yamakawa et al. (1991A) and two within their proximal deletion region whereas the fifth is located in 3p25. Thus far three genes on 3p have been proposed as candidate tumour suppressor genes: the acyl peptide hydrolase gene (APEH) (Erlandsson et al, 1990); the gene for protein-tyrosine phosphatase G (PTPG) (LaForgia et al., 1991), and a gene designated D8 (Carritt et al., 1992) which shows homology to the UBA1 gene coding for ubiquitin activating enzyme. (Kok et al, 1993). PTPG is most likely located in 3p14 (Drabkin et al., 1992). Densitometric analysis indicated loss of one allele in 3 of 5 RCC cell lines and in 5 of 10 lung cancer samples (LaForgia et al., 1991). A greatly reduced or undetectable level of PTPG expression was apparent in 9 of 31 lung cancer cell lines. However, a search for mutations in the coding sequences for one of the three domains of the PTPG gene was unsuccessful (Tsukamoto et al., 1992). Therefore, there is yet no proof that PTPG is a tumour suppressor gene. D8 and APEH are located 140 kb from each other in 3p21 (Carritt et al., 1992), most likely its distal part (Kok et al., 1987). No data are available, however, to draw the conclusion that D8 and APEH reside within the smallest common deletion in RCC as defined by Yamakawa et al. (1991a). We studied the expression of D8 and APEH in seven RCC cell lines and two RCC tumour samples (details in section 2.4). We found that the ranges of expression of both genes in RCC did not deviate from what was observed in normal kidney tissue. Therefore, we consider neither D8 nor APEH candidate tumour suppressor genes for RCC.

Although not as consistent as in RCC, lung cancer, and cervical cancer, occurrence of allelic losses on 3p is rather common in some other tumour types. However, in these tumours LOH on other chromosomes is as frequent or even more frequent than on chromosome 3. Examples are ovarian cancer (Ehlen and Dubeau, 1990, Sato et al., 1991a), endometrial cancer (Jones and Nakamura, 1992), breast cancer

(Devilee et al., 1989, Sato et al., 1990), testicular cancer (Lothe et al., 1989), and nasopharyngeal carcinoma (Huang et al., 1991). Jones and Nakamura (1992) made a deletion analysis of tumours of the uterine endometrium, uterine cervix, and ovary using CA-repeat polymorphisms. In three tumours of the uterine endometrium they found interstitial 3p deletions, with a common deletion region proximal of 3p21.3 and including a marker defining the locus D3S659 in 3p13 (localisation in Takahashi et al., 1992). In cancer of the uterine cervix four cases were found with interstitial 3p deletions, again including D3S659 in the region of common overlap. In ovarian cancer only one tumour with an interstitial 3p deletion was found. This tumour had a more distal deletion, including the locus D3S643 (3p21.3) which has also been found in the smallest common deletion region in RCC (Yamakawa et al., 1991a). In breast cancer, LOH analysis has revealed an interstitial 3p deletion in 10 tumours. The common deletion region appeared to be bordered by the loci D3S659 (3p13) and D3S678 (3p14.2-p14.3) (Sato et al., 1991b; loci mapped by in situ hybridization, Takahashi et al., 1992). This common deletion region in breast cancer is contained within the proximal common deletion region in RCC as found by Yamakawa et al. (1991a). Together, all these results from the literature leave the impression that, indeed, two regions, 3p14 and 3p21, may be involved in various tumour types. For sporadic RCC our own results point to a role of the 3p21 region. The 3p14 region will be discussed in relation with familial RCC in chapter 3.

Chromosome 3 is not the only chromosome for which allelic losses have been found in sporadic RCC. We studied loci on chromosomes 5, 11, 13, 17, and 22 which are often deleted in other types of tumours. We only found LOH on 17p, in 4 of 32 informative cases (see section 2.3). Bergerheim et al. (1989) studied LOH with one marker for every chromosome in 23 cases of RCC. Apart from LOH on chromosome 3 (15 of 22 informative cases or 68%) they found LOH on chromosome 18 in 5 of 15 informative cases. LOH on other chromosomes was less frequent. Morita et al. (1991a) studied LOH at loci on each arm of every autosomal chromosome -except 5p, 8p, and the short arms of the acrocentric chromosomes- in 38 cases of sporadic RCC. Allelic loss on 3p was the most frequent, in 14 of 22 informative cases (64%). On 16 chromosome arms the marker(s) used were informative in half or more of the cases. Of these chromosome arms 7q, 10q, and 19p showed LOH in 25% or more of the informative cases. Anglard et al. (1991) studied apart from chromosome 3 (LOH in

51 of 58 informative cases or 88%) LOH on chromosomes 11, 13, and 17. On chromosome 11 LOH occurred in 5 of 26 informative cases; on chromosome 13 in 3 of 13, and on chromosome 17 in 2 of 22. Thus, allelic losses on chromosomes other than #3 always occur at a much lower frequency than loss on 3p. It has been suggested that LOH on chromosomes other than 3 was primarily found in more advanced RCC (Morita et al., 1991b, Anglard et al., 1991, Presti et al., 1991) (see also chapter 4). This may indicate that an accumulation of allelic losses is associated with tumour progression. An inverse relationship between allelic losses on 3p and 17p in RCC was suggested by Ogawa et al. (1992). They found loss of 17p alleles in 6 out of 36 informative cases. In 5 of these cases also 3p was evaluated, and no allelic losses were found. In our material, however, case 16 shows allelic loss on 3p as well as on 17p (Fig.2). The results by Anglard et al. (1991) are also more indicative for an accumulation of allelic losses than for some exclusion. They found that the cases showing LOH on 17p also showed LOH on chromosome 13. The cases with LOH on chromosome 13 showed LOH on chromosome 11, and all cases with LOH on chromosome 11 showed LOH on chromosome 3. In this series concomitant loss at 3p and 17p occurs in 2 cases. Thus, both our results and those of Anglard et al. (1991) exclude a definite relationship as suggested by Ogawa et al. (1992). A kind of sequential order of involvement of chromosome regions in allelic losses seems apparent also in other types of tumour and might be related to some differential genomic instability (Buys, 1991).

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2.2 Direct molecular analysis of a deletion of 3p in tumors from patients with sporadic renal cell carcinoma

Direct Molecular Analysis of a Deletion of 3p in Tumors from Patients with Sporadic Renal Cell Carcinoma

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ABSTRACT: *Normal and tumorous nephrectomy specimens from seven renal cell carcinoma patients were subjected to a Southern analysis using chromosome #3-specific polymorphic probes. Three patients were not informative because of homozygosity at all loci studied. One patient showing heterozygosity at 3q in normal tissue had a tumor that remained heterozygous. In three patients the tumor showed loss of heterozygosity for a short arm marker at 3p21. In one of them heterozygosity for a second short arm marker was also lost. Another of these three patients retained heterozygosity for this second short arm marker, as well as for a long arm marker, suggesting a chromosomal breakpoint between the loci for the two short arm markers. Our results demonstrate that the known involvement of a short arm region of chromosome #3 in the development of renal cell carcinoma can readily be further evaluated by direct molecular methods.*

INTRODUCTION

Chromosomal rearrangements of the short arm of chromosome #3 have been described for both hereditary [1–3] and non-hereditary [4–7] forms of renal cell carcinoma (RCC). The chromosomal changes reported consist of translocations and deletions clustering in region 3p11-21. This region possibly contains a tumor suppressor gene or antioncogene [8] whose mutant or null alleles predispose to RCC. Functional loss of both normal alleles will lead to tumor development by the same mechanism as has been proposed for retinoblastoma and Wilms' tumor [9].

By in situ hybridization of single copy fragments of λCh4a-H3, a random human DNA clone detecting a polymorphic HindIII site on chromosome #3 [10], we recently assigned this polymorphism to 3p21 [11]. A subclone pH3H2, also detecting this polymorphic site, was used to investigate the occurrence of possible deletions of 3p21 directly into renal cancer DNA. This was done by monitoring loss of heterozygosity in renal cell tumors of patients that had heterozygous patterns in their constitutive DNA. We previously used the same approach, applying the same probe,

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in small cell lung cancer [12] where 3p deletions are also a consistent abnormality [13, 14]. In this study, we also included some other chromosome #3-specific probes.

MATERIALS AND METHODS

High molecular weight DNA was isolated from tumor tissue and normal kidney tissue from the same patient obtained by nephrectomy. The diagnosis of RCC was based on histologic investigation. All cases were sporadic ones. Samples were minced and incubated in 10 mM Tris HCl, pH 7.0, 1.0 mM EDTA, 100 mM NaCl, proteinase K 50 μg/ml, 0.5% SDS, at 37°C for 18 hours. After phenol/chloroform extractions and isopropanol precipitation, DNA was dissolved in 10 mM Tris HCl, pH 7.5, 0.1 mM EDTA.

DNA was digested with restriction enzymes under conditions recommended by the supplier (BRL). Agarose gel electrophoresis was carried out at low voltage using 0.7% gels. Transfer to Gene Screen Plus membrane (New England Nuclear) was performed in 0.4 M NaOH, 0.6 M NaCl, for 24 hours. Filters were prehybridized in 0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 1.0 mM EDTA, 1.0% bovine serum albumin, at 65°C for 1–2 hours [15]. Probes were labeled by nick-translation to a specific activity of approximately 10^8cpm/μg. Per 15 ml prehybridization solution 100 ng denatured labeled probe was added. Hybridization was carried out at 65°C overnight. Filters were washed at room temperature in 40 mM $NaHPO_4$, pH 7.2, 5% SDS, 1.0 mM EDTA, 0.5% bovine serum albumin, for 5 minutes, followed by three washes of 5 minutes each in the same solution without serum albumin. Fuji RX X-ray film was exposed at −80°C for 18 hours up to 9 days. Autoradiographs were scanned with a Joyce Loebl microdensitometer.

RESULTS AND DISCUSSION

DNA was extracted from nontumorous parts of nephrectomy specimens obtained from seven different patients (A–G). Digestions were carried out with the restriction endonucleases HindIII and MspI, allowing the detection of restriction fragment length polymorphisms for pH3H2 (DNF15S2) and pHS3 (D3S1), and for p12-32 (D3S2) and pHS1-37 (D3S3), respectively. The probe p12-32 is localized at 3p [16], pMS1-37 at 3p14 [17], and pHS3 at 3q12 [18]. A Southern analysis of the constitutive DNA revealed heterozygosity for pH3H2 in patients A, B, and C, for p12-32 in patients A and C, and for pHS3 in patients C and F (Table 1). Probe pMS1-37 was not informative for any of these patients. Tumor DNA from these patients was subjected to the same analysis. All patients that appeared to be heterozygous for pH3H2 in their normal tissue showed a different pattern in their tumor tissue (Fig. 1). Although two bands were visible on the autoradiograph, they showed a marked difference in intensity. The intensity ratios between the higher band representing the 2.3-kb allele and the lower one representing the 2.0-kb allele were 1.8 for patient A, 2.8 for patient B, and 2.9 for patient C. Because in blood samples of true heterozygotes the relative intensities as measured under our conditions of hybridization and autoradiography vary between 0.9 and 1.2 (14 samples), the presence of the second fainter band must be due to cellular heterogeneity, most probably admixture of normal cells in the tumor samples. A similar loss of heterozygosity was also observed with p12-32 in patient A (autoradiograph not shown). In patient C loss of heterozygosity was observed for pH3H2, whereas, for p12-32 and pHS3 heterozygosity is retained in the tumor. This suggests a breakpoint between the loci for pH3H2 and p12-32 on the short arm of chromosome #3. The localization of p12-32 might therefore be crucial to determine the subregion involved in the development of RCC.

Table 1 Alleles in normal (N) and tumor (T) tissue of nephrectomy specimens from renal cell carcinoma patients

Patient	Tissue	Probe: pH3H2	pMS1-37	p12-32	pHS3
A	N	1/2[a]	—	1/2	—
	T	1	—	2	—
B	N	1/2	—	—	—
	T	1	—	—	—
C	N	1/2	—	1/2	1/2
	T	1	—	1/2	1/2
D	N	—[b]	—	—	—
	T	—	—	—	—
E	N	—	—	—	—
	T	—	—	—	—
F	N	—	—	—	1/2
	T	—	—	—	1/2
G	N	—	—	—	—
	T	—	—	—	—

[a] Of each pair of alleles the larger fragment has been designated 1, the smaller 2.

[b] DNA was found not to be heterozygous.

Our results clearly show that it is possible to demonstrate the occurrence of 3p deletions in RCC by a direct DNA analysis of tumor tissue compared with constitutive tissue. Obviously, this approach is much easier than conventional karyotyping, especially because it does not require culturing of tumor cells or chromosome spreads to be prepared directly from solid tumor material. During the preparation of this manuscript a comparable study appeared describing a larger group of patients [19]. Taking the number of patients from that and our study together 14 of 25 patients could be evaluated for loss of heterozygosity of 3p. The frequency of het-

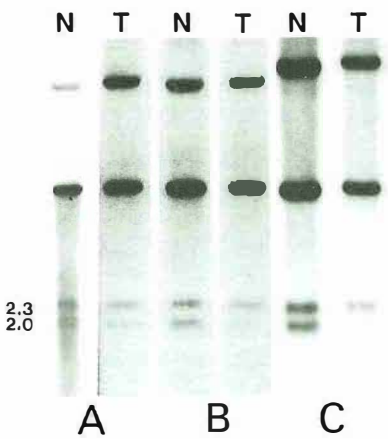

Figure 1 Comparison of hybridization patterns of HindIII restricted DNA from normal and tumorous kidney tissue of three RCC patients (A, B, and C) at the 3p21 locus recognized by pH3H2. Bands at 2.3 kb and 2.0 kb represent a polymorphism on chromosome #3, the two longer bands represent fragments on chromosome #1 [10, 20]. Note the marked difference in intensity between the 2.3-kb and the 2.0-kb bands in the tumor patterns.

erozygotes detectable with the available 3p probes puts a limit to the proportion of patients that can be investigated by DNA analysis. More polymorphic probes from the 3p region will certainly become available in the immediate future. This will allow a molecular analysis of virtually all patients, and may soon reveal which region represents the minimal common deletion in RCC, thus narrowing the region containing a possible tumor suppressor gene whose mutant alleles are involved in the development of this cancer. This might also answer the question whether the same gene or a different one is involved in lung cancer [11].

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2.3 The region of common allelic losses in sporadic renal cell carcinoma is bordered by the loci D3S2 and THRB

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The Region of Common Allelic Losses in Sporadic Renal Cell Carcinoma Is Bordered by the Loci D3S2 and THRB

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Cytogenetic studies and DNA analysis have shown that the short arm of chromosome 3 is the region in the genome that is commonly deleted in renal cell carcinoma. By studying loss of heterozygosity in 41 matched tumor/normal kidney tissue pairs, we could delimit the commonly deleted part of 3p to the region between the loci THRB (in 3p24) and D3S2 (in 3p21). The regions on 3p suggested to be involved in the Von Hippel–Lindau syndrome and in hereditary renal cell carcinoma are both outside this smallest region of overlapping deletions. Consequently, renal cell cancer would be an illustration of the possibility that different genes cause the same type of tumor. © 1991 Academic Press, Inc.

INTRODUCTION

Renal cell carcinoma (RCC) is the most common tumor of the kidney in adults. Most cases are sporadic, but familial cases have also been described, both as part of the Von Hippel–Lindau syndrome and without any association with this syndrome.

Cytogenetic studies have shown that the short arm of chromosome 3 is the region of the genome most commonly deleted in renal cell carcinoma (Yoshida *et al.*, 1986; Kovacs and Frisch, 1989; De Jong *et al.*, 1988; Walter *et al.*, 1989). The most frequent chromosomal abnormalities resulting in the loss of genetic material from 3p are terminal deletions and unbalanced translocations with breakpoints in the region 3p11–3p21.

This involvement of a specific chromosomal region in both familial (Cohen *et al.*, 1979; Pathak *et al.*, 1982) and sporadic RCC is consistent with the tumor suppressor gene model (Knudson, 1985; Hansen and Cavenee, 1987). Such a tumor suppressor gene may be a differentiation gene coding for a product that suppresses proliferation. Mutant alleles may become unmasked by loss of the normal allele at the homologous chromosome, resulting in proliferation and tumor formation.

Indeed, DNA analysis of RCC has demonstrated loss of heterozygosity at certain loci on 3p. Whereas most cases showed loss of heterozygosity at one or more loci on 3p (Zbar *et al.*, 1987; Van der Hout *et al.*, 1988; Kovacs *et al.*, 1988; Bergerheim *et al.*, 1989), some had a retention of heterozygosity at all informative loci. In this study of 41 cases we confirm that in a substantial percentage of cases deletions are either absent or too small to be detected. We could delimit the commonly deleted region by using more RFLPs in the region 3p21–p25.

MATERIALS AND METHODS

High-molecular-weight DNA was isolated from 41 tumors and matched normal kidney tissue from 39 patients. All tumors were primary renal cell carcinomas, except tumor sample 35, which was a lung metastasis from primary tumor 22; tumor sample 26, which was a bone metastasis; and tumor samples 45 and 54, which were lymph node metastases from primary tumors 46 and 53, respectively. Thirty-seven tumors were collected in Groningen, and 4 in Boston. Most tumor samples used for DNA isolation contained at least 75% tumor cells according to histological evaluation. Only tumor samples 31 and 41 had between 50 and 75% tumor cells and tumor sample 27 had between 25 and 50%.

DNA extraction, Southern analysis, and autoradiography were carried out as described earlier (Van der Hout *et al.*, 1988). Probes were labeled by random oli-

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TABLE 1

Chromosome 3 Probes Used in RFLP Analysis

Locus	Probe	Localization	Refs.
RAF1	p628	3p25	(14)
RARB	pcod20	3p24	(5, 14)
THRB	pBH302	3p24	(1, 14)
	pHeA2		
D3F15S2	pH3H2	3p21	(14)
D3S48	c13B5.1[a]	3p21	
D3S2	pHF12-32	3p21	(14)
D3S6	DR82	3p13	(1, 14)
D3S3	pMS1-37	3p14	(1, 14)
D3S1	HS3	3q12	(14)
MOX2	HX2/HIII	3q12-14	(14, 22)
D3S5	DR2	3q25-28	(14, 29)

[a] For c13B5.1, which was recently isolated in our laboratory, full data will be published elsewhere.

gonucleotide priming (Feinberg and Vogelstein, 1983). The studied loci on chromosome 3 and the corresponding DNA probes used are listed in Table 1. To study loci outside chromosome 3 often deleted in other types of tumors we used probes from the following loci: D5S6 (5q22–31), INS and HRAS (11p15), RB1 and D13S1 (13q14), D17S5 (17p13), and IGLV and IGLC (22q11).

RESULTS

DNA was isolated from 41 tumor specimens and matched normal kidney tissue. Parallel samples were analyzed with 12 probes that detect 16 RFLPs on chromosome 3. One case was uninformative at all loci. The pattern of allelic losses in the remaining 40 tumors is given in Table 2. A total of 16 of the informative cases showed no allelic loss at any of the loci studied. For 20 patients all informative loci at 3p (varying in number from 1 to 5) showed loss of heterozygosity, and 4 cases (16, 43, 48, 49) showed loss of heterozygosity at some but not all of their informative loci at 3p. These 4 cases are illustrated in Fig. 1. In cases with unequal band intensities in the tumor lanes, we had to distinguish between two possibilities: either the fainter band is caused by an admixture of normal tissue in the tumor, or the darker band is due to the presence of an extra copy of that allele in the tumor. This problem may be solved by rehybridization of the filters with a probe derived from a different chromosome not showing a frequent loss of heterozygosity. In a series of 13 cases informative for chromosome 13-derived probes, we never found loss of heterozygosity. Therefore, a chromosome 13-specific probe (D13S39) was used to rehybridize those filters that showed unequal band intensities in the tumor DNA lanes when hybridized with a polymorphic chromosome 3 probe. If the darker band is caused by the presence in tumor cells of an extra copy of one of the chromosome 3 alleles, the ratio of the band intensities of the other allele in the normal DNA and tumor DNA lanes and the intensity ratio of the bands caused by the chromosome 13-specific probe in the normal DNA and tumor DNA lanes, respectively, will be equal. If these ratios differ significantly, this must be due to some admixture of normal cells. The longer alleles of D3F15S2 and D3S2 in DNA from tumor 49 and of pH3H2 in DNA from tumor 48 could only be explained by an admixture of normal tissue in the tumors. Tumor

TABLE 2

Loss of Heterozygosity on Chromosome 3 in RCC

	Locus								
Tumor	RAF1 3p25	RARB p24	THRB p24	D3F15S2 p21	c13B5.1 p21	D3S2 p21	D3S6 p14	D3S3 p14	3q[a]
1			x	x					
3	x		x			x			0
4			x						0
5		0	0	0					
6	x			x	x	x			0
7	0		0						
8	0		0	0					0
11			x			x			
15		x	x	x					0
16	0		x						0
17			0		0	0			0
22	x		x						0
35	x		x						0
23			0	0	0	0			0
24			0			0	0	0	0
26	x					x			0
27	0		0				0		0
29			x						0
30			0	0					0
31			0		0				0
33						x			0
34	x	x	x	x			x		
36	x		x	x		x			
37	0	0							0
38				x	x				
40	0				0				0
41	0		0	0		0			
42		x	x			x		x	
43			x	x				0	0
45	x		x	x	x				
46	x		x	x	x				
47	x		x	x					
48	0		0	x					
49	0		0	x		x			0
50	0		0	0	0				
52	0		0						
53			x	x					x
54			x	x					x
55	0		0	0		0			0
56			0			0			

Note. x, loss of heterozygosity; 0, no loss of heterozygosity; no entry: homozygous in normal tissue.

[a] Data for the three different 3q probes have been linked.

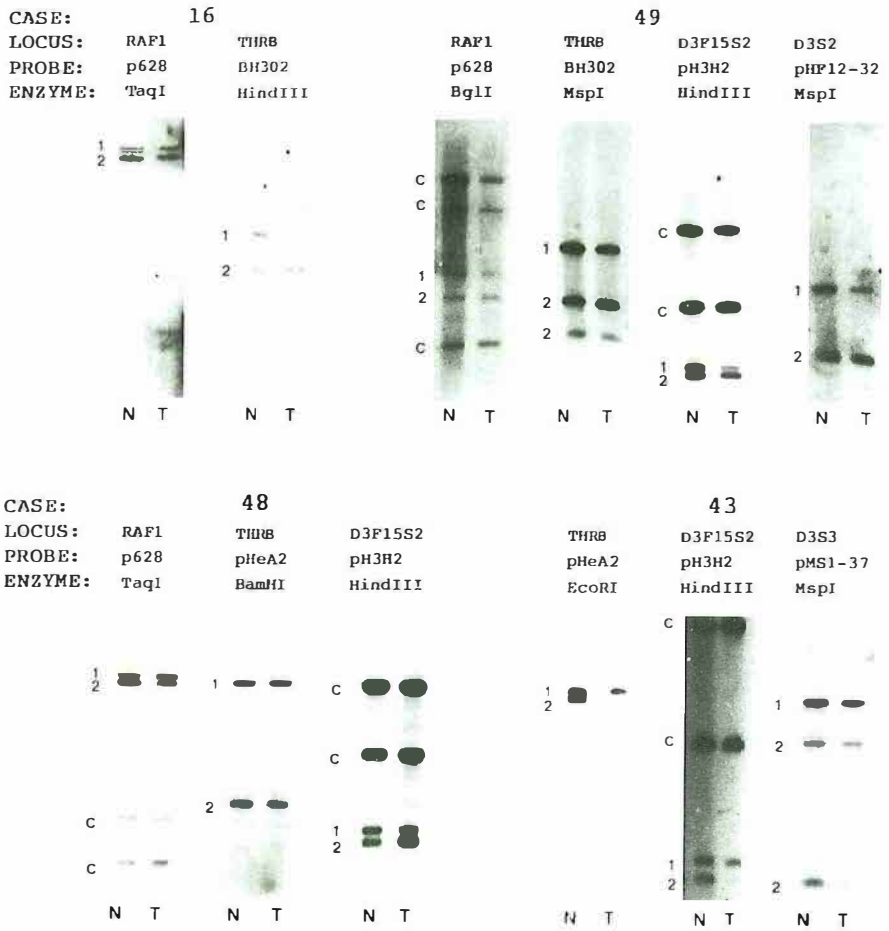

FIG. 1. Southern hybridizations of 3p probes to normal (N) and tumor (T) DNA in four cases. For each RFLP the longest allele is marked 1 and the shortest 2. Constant bands are marked c. Loss of heterozygosity for BH302 and retention for p628 are shown for tumor 16, loss of heterozygosity for pH3H2 and pHF12-32 and retention for p628 and BH302 for tumor 49, loss of heterozygosity for pH3H2 and retention for p628 and pHeA2 for tumor 48, and loss of heterozygosity for pHeA2 and pH3H2 and retention for pMS1-37 for tumor 43. The presence of the less intense bands for allele 1 of pH3H2 and allele 1 of pHF12-32 in tumor 49 and allele 1 of pH3H2 in tumor 48 must be due to admixture of normal tissue.

DNA of case 43 was virtually free of contaminating normal DNA, as can be concluded from the absence of one of the pHeA2 or pH3H2 bands in the lanes with tumor DNA. Thus, the presence of both alleles of D3S3 in tumor 43 must reflect the retention of heterozygosity at this locus. Figure 2 shows the possible deletions in tumors 16, 43, 48, and 49. From the literature several cases that show retention of heterozygosity at the locus D3S2 and allelic loss at D3F15S2 are known (Kovacs *et al.*, 1988; Zbar *et al.*, 1987). These cases have also been included in Fig. 2. A smallest common deletion comprising the region bordered by THRB (3p24) and D3S2 (3p21) can thus be deduced.

In 24 tumors at least one of the 3q probes used was informative. Heterozygosity of 3q probes was retained in all cases except case 53 (and its metastasis, 54) (Table 2). Therefore, in Fig. 2 retention of heterozygosity for 3q loci is assumed when the 3q probes were uninformative.

In the two cases where we could study the primary tumor as well as a metastasis, the pattern of allelic

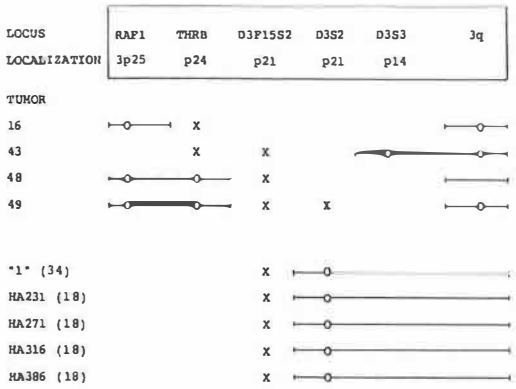

FIG. 2. Schematic representation of partial deletions of 3p. Interruptions of the line representing chromosome 3 give the maximal size that can be presumed for the deletion. X, loss of heterozygosity; 0, no loss of heterozygosity. Data used are those from Fig. 1. Moreover, some cases from the literature have been added. 1 is patient 1 from Zbar *et al.* (15) and HA231, HA271, HA316, and HA386 are four cases from Kovacs *et al.* (18).

losses on chromosome 3 was the same for the tumor and the metastasis.

For chromosomes 5, 11, 13, 17, and 22 the number of informative cases studied for loss of heterozygosity was 9, 14, 13, 32, and 8, respectively. Except for four cases with loss of heterozygosity on 17p (tumors 16, 17, 30, and 41) no losses were found.

DISCUSSION

The smallest deletions in RCC as determined by loss of heterozygosity have so far been described by Bergerheim *et al.* (1989). They reported terminal deletions comprising the major part of the short arm of chromosome 3 from the very end to some breakpoint between D3S6 in 3p14 and D3S2 in 3p21. In their material no evidence of interstitial deletions was found. In two tumors that karyotypically also seemed to have terminal 3p deletions, Teyssier *et al.* (1986), however, showed by *in situ* hybridization that the subterminal RAF1 locus in band 3p25 was still present on the chromosome 3 homologue with the deletion.

We have found four cases of RCC in which loss of heterozygosity data indicates a partial deletion of 3p. The smallest common deletion in these four cases is the region bordered by the loci D3S3 and THRB. If we combine our data with those from other studies (Kovacs *et al.*, 1988; Zbar *et al.*, 1987), the smallest region of overlap of 3p deletions in sporadic RCC is the region between D3S2 (3p21) and THRB (3p24). This region has also been reported as the common deletic region in lung cancer (Naylor *et al.*, 1987; Brauch *al.*, 1987; Kok *et al.*, 1987). For the moment there a no arguments that favor the involvement of eith one or different genes in RCC and lung cancer.

The circumstance in which we find a relativel large number of tumors without any loss of heterozy gosity may well be due to a lack of probes in the rele vant region. Another explanation is heterogeneit within RCC.

Unlike some other tumor types (Yokota *et al.*, 1987 Scheffer *et al.*, 1991) RCC does not show multiple al lelic losses at a number of chromosomes. Loss of het erozygosity is restricted mainly to chromosome 3. A gene on (the short arm of) chromosome 17 may play some additional role in the development of RCC, since this was the only other region showing allelic loss. The p53 gene might be a candidate for this. Loss of an entire copy of chromosome 3 does not seem a frequent mechanism for inducing RCC tumor formation. Only in one case (tumor 53 and its metastasis tumor 54) did we found loss of heterozygosity at 3p and concomitant loss at 3q.

It has been suggested that sporadic RCC and the Von Hippel–Lindau syndrome (VHL) are caused by mutations of the same gene at 3p. The latter syndrome is an autosomal dominantly inherited disorder predisposing for various forms of cancer of which RCC is one of the more frequent. Karyotypic abnormalities of 3p have also been found in RCC from VHL patients (King *et al.*, 1987; Decker *et al.*, 1989; Jordan *et al.*, 1989; Goodman *et al.*, 1990). In one RCC tumor from a VHL patient loss of heterozygosity at D3F15S2 was found (Decker *et al.*, 1989). Close genetic linkage has been described between VHL and the RAF1 oncogene at 3p25 (Seizinger *et al.*, 1988; Vance *et al.*, 1990). Based on multipoint linkage analysis the most likely location for VHL is in the interval between RAF1 (3p25) and D3S18 (3p26) (Hosoe *et al.*, 1990). Our finding that the smallest common deletion in sporadic RCC lies between D3S2 and THRB makes it more likely that sporadic RCC and VHL are caused by mutations of different genes.

In a family with hereditary RCC, Cohen *et al.* (1979) found a constitutional t(3;8) in all family members who had inherited the disease. The translocation breakpoint on chromosome 3 was at 3p14 (Wang and Perkins, 1984). In another RCC family described by Pathak *et al.* (1982) a t(3;11) was found in a tumor. Again, the breakpoint on chromosome 3 was in the same region (3p13–14). It has been speculated that one of the copies of a tumor suppressor gene is (functionally) eliminated as a consequence of these translocations. In the familial t(3;8), the breakpoint is flanked by the loci D3S3 and D3S2 (Gerber *et al.*, 1986; Van der Hout *et al.*, 1991). This implies that, if

ıe speculation is correct, a gene for heritable RCC ould be located proximal to the region harboring the ene for sporadic RCC. Consequently, renal cell ancer would be an illustration of the possibility that ifferent genes cause the same type of tumor, in the ase of RCC occurring either sporadically, or in fami- es, as pure RCC or as part of the VHL syndrome.

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2.4 A gene in 3p21 with greatly reduced expression in lung cancer, is well-expressed in renal cell carcinoma

(submitted for publication)

A gene in 3p21 with greatly reduced expression in lung cancer, is well-expressed in renal cell carcinoma.

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Abstract

In both lung cancer and renal cell cancer (RCC) 3p21 is a common region of deletions. A gene from this region (designated *D8*) was found to be not expressed or dramatically underexpressed in cell lines of all types of lung cancer. We studied the expression of this candidate tumour suppressor gene in RCC. Both in the RCC cell lines and in the RCC tumours studied the expression of *D8* appeared to be at a level comparable to or higher than that in normal kidney cells. The acylpeptide hydrolase gene (*APEH*) is also located in the commonly deleted region. A substantial reduction of the expression of this gene in RCC tumours has been reported. In our material, however, we could not confirm this. Probes from the *D8* and *APEH* loci did not detect genomic alterations in RCC. Therefore, we see no evidence for a role of either *D8* or *APEH* as a tumour suppressor gene in RCC.

Introduction

A gene -provisionally designated *D8*- in band p21 of chromosome 3 appears to be well-expressed in normal adult lung but to be consistently and specifically not expressed or dramatically underexpressed in a variety of lung cancer cell lines (Carritt et al., 1992, Kok et al., 1993). Because of both its location in the region commonly deleted in lung cancer (Kok et al., 1987) and its greatly reduced expression, *D8* might be considered as a candidate lung cancer tumour suppressor gene. In renal cell cancer (RCC), the commonly deleted region according to our data (van der Hout et al., 1991) includes the *D8* locus. This

might also be the case for the data of Yamakawa et al. (1991) that delimit the common deletion to an even smaller region. Therefore, we wondered whether the expression of the *D8* gene would also be reduced in RCC. We included in our studies the acylpeptide hydrolase gene (*APEH*) (Erlandsson et al., 1991), located 140 kb from *D8* (Carritt et al., 1992), as it has been reported that the expression of *APEH* was greatly reduced in RCC tumours (Erlandsson et al., 1990).

Materials and methods

Cell Cultures

RNA was isolated from 6 established RCC cell lines (RCC1, RCC4, RCC5, RCC6, RCC7, RC21), 2 RCC tumour samples (HA277 and T1748), 1 small cell lung cancer (SCLC) cell line (GLC2), and 5 short term cultures of normal renal tissue from RCC patients (MN3, MN6, MN8, MN9, and MN10). All RCC cell lines and tumours were of the clear cell type, except for RCC5 which was derived from a chromophilic tumour. RC21 was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The other RCC cell lines (RCC1, RCC4, RCC5, RCC6 and RCC7) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 0.5mM arginine, and 0.5mM asparagine. The SCLC cell line was cultured in RPMI 1640 medium supplemented with 15% fetal calf serum, $5x10^{-5}$M ß-mercaptoethanol, 1mM sodium pyruvate, and 50µg/ml gentamycin. Normal renal tissue was disaggregated and cultured for 4 passages at the most, in DMEM supplemented with 10% fetal calf serum. From the disaggregated tissue cells grew out with a morphology similar to that of the cells of the RCC cell lines (triangular to roundish cells, they did not look like fibroblasts). Patients from whom normal renal tissue was obtained were different from those whose tumours had been used to generate the RCC cell lines.

RNA Isolation

Total RNA from tissue culture cells was isolated using guanidine hydrochloride (Kok et al., 1993). To isolate RNA from frozen tumour tissue we applied a method using LiCl and urea (Auffrey and Rougeon, 1980).

Probes

The probes we used were a 3.3kb cDNA fragment of *D8* (Carritt et al., 1992), a genomic clone from the *APEH* locus, H3E4 (Naylor et al., 1989), and a hamster ß-actin c-DNA (Dodemont et al., 1982). Probes were labeled by random oligonucleotide priming (Feinberg and Vogelstein, 1983).

Northern Analysis

Approximately 15µg RNA per lane was resolved on a 1% agarose gel containing 2.2M formaldehyde. RNA was transferred to Hybond N^+ membranes (Amersham International, Amersham, U.K.). Hybridization was carried out in 0.5M $NaHPO_4$, 7% SDS, 10mM EDTA, and 1% bovine serum albumin, pH7.2, at 67°C, overnight. Post hybridization washes were also carried out at 67°C.

Southern Analysis

DNA was isolated from cultured cells and tumour samples and subjected to Southern analysis using the same *D8* and *APEH* probes as in the Northern analysis according to methods published elsewhere (Van der Hout et al., 1991).

Analysis of microsatellite polymorphisms

CA-repeat markers CI3-9 and CI3-830, located in 3p21.3 and 3p21.3-22 respectively (Jones et al., 1992), were investigated in DNA from the RCC cell lines following the method described by Jones et al. (1992) with a small modification: we used 300 ng DNA and cycled the PCR 25x.

PCR-aided transcript titration assay

This assay was carried out according to Kok et al. (1993).

Results

Hybridization of *Eco*RI digested DNA of RCC cell lines with probes from the *D8* and *APEH* loci did not reveal any genomic rearrangement. Analysis of two CA-repeat polymorphisms in the region 3p21.3-p22 in these cell lines revealed in each case only a single band.

Total RNA was isolated from 6 RCC cell lines, 2 primary RCC tumours, 1 SCLC cell line, and 5 short term cultures of normal kidney tissue. Two identical agarose gels were run and Northern blots were sequentially hybridized with the *D8* probe and ß-actin or with the *APEH* probe and ß-actin. In these hybridizations ß-actin served as a standard of RNA loading and integrity. The results of these Northern analyses are shown in Fig.1. It appears that the variation in the expression level of both *D8* and *APEH* in the RCC cell lines and tumours does not differ from the variation observed in the normal kidney samples. An exception is

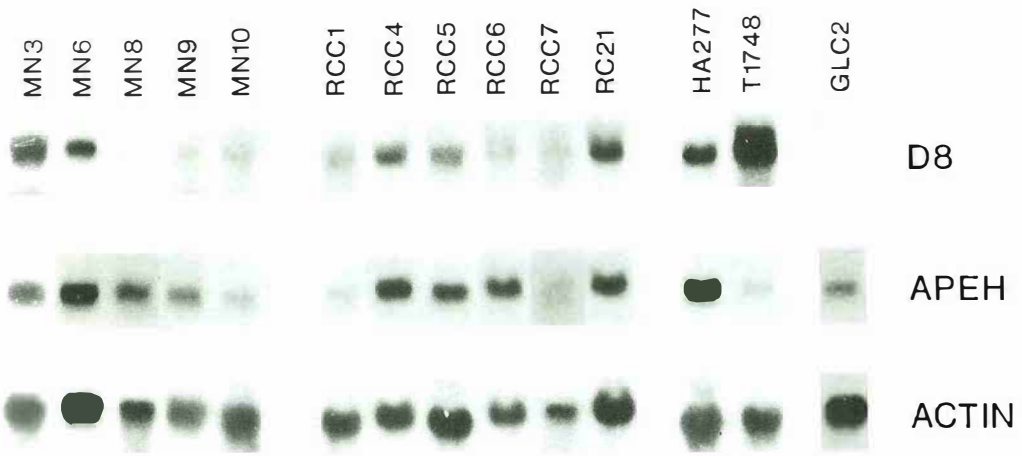

Fig.1. Expression of the D8, APEH, and ß-actin genes in normal kidney tissue (MN3, MN6, MN8, MN9, MN10), RCC cell lines (RCC1, RCC4, RCC5, RCC6, RCC7, RC21), RCC tumour samples (HA277, T1748) and an SCLC cell line (GLC2) as analyzed by the Northern blotting procedure.

tumour sample T1748 which shows a very high expression of *D8*. As found earlier for a collection of 23 SCLC cell lines (Carritt et al., 1992), Northern analysis did not reveal any *D8* expression in the SCLC cell line GLC2 which we included here for comparison.

Discussion

Since we had not available DNA from normal tissue of the individuals from whom the RCC cell lines derived, a direct analysis of loss of heterozygosity was excluded. Therefore, we analyzed two microsatellite polymorphisms with heterozygosity values of 0.77 and 0.70, respectively (Jones et al., 1992). There is a probability of 0.069 ((1-0.77)(1-0.70)) that an individual would turn out not to be heterozygous at all with these markers. For 6 out of 6 individuals to show a homozygous pattern of hybridization means a probability of $(0.069)^6$ i.e. about 10^{-7}. Our finding of a single hybridization signal in all cases indicates that loss of heterozygosity must have occurred in at least some of the RCC cell lines. For one of the two RCC tumour samples included in this study loss of heterozygosity on 3p has been demonstrated (T1748 = case 22 in Van der Hout et al., 1991). For the other tumour, HA277, matching normal DNA as well as tumour DNA was not available. Thus, for at least a subset of the tumour material studied here, the region of the *D8* and *APEH* genes was present in a single copy. We found no indications for rearrangements in the remaining copy.

Whereas in all 23 SCLC cell lines examined for *D8* expression in a previous study (Carritt et al., 1992) transcripts were undetectable by Northern analysis, and a PCR-aided transcript titration assay showed that in most lung cancer derived cell lines *D8* expression is only 2% or less of that in normal lung tissue (Kok et al., 1993), our present results show that in RCC cell lines and tumours the expression of *D8* is normal, in one case even elevated. In a quantitative transcript titration assay an expression level of 40 copies of *D8* messenger per 10 pg cellular RNA in both cell line RCC7 and normal kidney MN2 has been measured (results not shown). In lung tumours expression was between 0.2 and 1.4 copies *D8* messenger per 10 pg cellular RNA (Kok et al., 1993). Thus,

in case *D8* may be attributed any role in the development of lung cancer because of its very low expression, this does not hold for the development of RCC.

There are other genes in this region that have been reported to have a low expression in RCC. By a semi-quantitative Northern analysis in 15 RCC tumours it was found that the expression of the *APEH* gene was reduced to less than 20% of the expression in normal kidney in 11 cases and that it was at the most 51% of the normal value in the remaining 4 cases (Erlandsson et al., 1990). In our material, however, the range of *APEH* gene expression in RCC cell lines and tumour samples as assessed from Northern analysis did not deviate from that in normal renal tissue. A reduced expression has been reported for a protein-tyrosine phosphatase gene (*PTPG*) but in only some (3 out of 5) of the RCC cell lines studied (LaForgia et al., 1991). A search for mutations in the *PTPG* gene has been unsuccessful until now (Tsukamoto et al., 1992). Therefore, there is yet no indication that these genes fulfil the role of tumour suppressor genes. A further delimitation of the 3p region of common deletions in RCC along the lines we recently indicated (Van der Hout et al., 1993) will be necessary to define new candidate tumour suppressor genes for RCC.

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CHAPTER 3

Involvement of the chromosome region 3p14 in renal cell carcinoma

3.1 Introduction

In a family with pure familial RCC reported by Cohen et al. (1979) a balanced constitutional translocation (3;8)(p14.2;q24.1) was found segregating with the tumour (c.f. section 1.2.1). It has been speculated that this inherited translocation may represent the first (germline) mutation of Knudson's two-hit hypothesis. This would imply the presence of a tumour suppressor gene in the chromosome region affected by the translocation. It is the involvement of chromosome 3 and not of chromosome 8 which seems to be relevant in the translocation (3;8) since chromosome 3 is involved in all forms of RCC (c.f. sections 1.1.2 and 1.2.2). Notably, the same region 3p14 is known as a second common deletion region in sporadic RCC (Yamakawa et al., 1991, discussed in section 2.1). The chromosome 8 segment involved in the t(3;8) has received some attention because the oncogene c-myc, located in 8q24, is transferred to the der(3) chromosome (Drabkin et al., 1985). However, a carrier of the t(3;8) showed no alterations of myc expression nor any genomic rearrangement in a region extending from 1000 kb to the 5' side to 500 kb to the 3' side of c-myc (Gemmill et al., 1989). A common deletion region in 3p proximal to 3p21 has also been found in some other tumour types. In tumours of the endometrium the common deletion region, bordered by the loci D3S1079 in 3p13 and D3S1029 in 3p21.2-p21.3 includes the t(3;8). This might also be the case in tumours of the uterine cervix (common deletion region between D3S1136 in 3p13 and D3S1228 in 3p14.1-p14.3) (Jones and Nakamura, 1992). Cloning of the immediate surroundings of the breakpoint in RCC may provide an opportunity to establish whether the translocation has affected a gene possibly involved in the development of familial RCC and some other tumour types.

It may be noted, however, that in breast cancer a common deletion region has been found between D3S659 (3p13) and D3S678 (3p14.2-p14.3) (Sato et al., 1991) which are both located proximal to the t(3;8) according to Yamakawa et al. (1991). Thus, another tumour suppressor gene may be located more proximal.

In order to localize three widely used 3p probes with respect to the (3;8) breakpoint, we hybridized them in situ to metaphase chromosomes of a carrier of the t(3;8). The probe defining the locus D3S3 did not hybridize to either of the two translocation chromosomes, although Southern analysis indicated that D3S3 was present on the der(3) chromosome carrying the 3qter-p14 part. We tentatively concluded that D3S3 might be close to the 3p14 breakpoint and might be rendered inaccessible because of distortion of the higher order chromatin structure by the translocation. The detailed analysis is given in section 3.2.

Because of this possible location of D3S3 in the vicinity of the t(3;8) breakpoint we decided to map the region around this locus. In section 3.3 the construction of a long range restriction map is described. Because the D3S3 region turned out to be heavily methylated which severely hampers map construction, we tried to obtain partially demethylated DNA by isolating it from cells grown in a medium containing the demethylating agent 5-azacytidine. Using this partially demethylated DNA a map of approximately 1 Mb around D3S3 could be made. Comparison with restriction fragments from DNA of the translocation carrier yielded no indication that the breakpoint is to be found within the mapped area.

To map a larger part of 3p14 we needed many more probes from this band. A direct way to get these is microdissection of the relevant band from metaphase chromosomes followed by cloning of the dissected material. Section 3.4 describes the characterization of a 3p14 specific microdissection library and the use of this library in mapping the region around the t(3;8) breakpoint.

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3.2 Localization by in situ hybridization of three 3p probes with respect to the breakpoint in a t(3;8) in hereditary renal cell carcinoma

Localization by In Situ Hybridization of Three 3p Probes with Respect to the Breakpoint in a t(3;8) in Hereditary Renal Cell Carcinoma

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ABSTRACT: *Three 3p probes were localized by in situ hybridization on chromosomes from a carrier of a balanced t(3;8) associated with renal cell cancer. For one of the probes (pH3E4/D3S48), a previous localization in 3p21 was confirmed; for a second probe (pHF12–32/D3S2), a broader localization could be confined to 3p21. Both probes appeared to be located distal to the breakpoint in 3p. The third probe (pMS1–37/D3S3) was localized to 3p14, in accordance with a previous localization. This probe, however, hybridized very weakly or not at all to either of the translocation products, although it is known from Southern analysis that the D3S3 sequence is present on one of them. We assume that this probe is located close to the breakpoint on 3p and that distortion of the higher-order chromosomal structure in this region is causing the failure of the in situ hybridization.*

INTRODUCTION

In renal cell carcinoma (RCC), frequent losses of a substantial part of the short arm of chromosome 3 have been microscopically observed [1–6]. At the molecular level, loss of heterozygosity has been found for several 3p probes [4, 7, 8]. It has been suggested that development of renal cell cancer is due to loss of a recessive tumor suppressor gene on 3p. Age-incidence curves of hereditary and nonhereditary cases are in agreement with this suggestion [9].

A family has been reported in which a balanced constitutional t(3;8) is present in eight family members who developed renal cell cancer. Initially, the breakpoint in chromosome 3 was assigned to band p21 [10]. Results of a later study using prometaphase analysis indicated that the breakpoint is at band p14.2 [11]. Carriers of the t(3;8) would have one copy of the putative tumor suppressor gene already eliminated or inactivated by the translocation [12]. This would imply that the tumor suppressor gene is located at the translocation breakpoint or very close to it. For a closer approach of this presumed gene, we would like to know the location of available 3p probes with respect to the chromosomal breakpoint. We localized three 3p probes on the translocation products by in situ hybridization and could simultaneously verify the localization of these probes on the normal copy of chromosome 3.

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MATERIALS AND METHODS

Metaphase chromosome spreads were prepared from skin fibroblasts of a carrier of the balanced translocation t(3;8) from the family described by Cohen et al. [10]. Cell culture, preparation of metaphase chromosome spreads, and in situ hybridization methods have been described previously [13]. We used the probes pH3E4/D3S48 [14], pHF12–32/D3S2 [15], and pMS1–37/D3S3 [16].

RESULTS

Following the in situ hybridization, the location of grains for each of the three probes pH3E4, pHF12–32, and pMS1–37, on the normal copy of chromosome 3 and on the two translocation chromosomes, der(3) and der(8), was recorded for 160–220 well-banded metaphases. Approximately one out of four metaphases showed a grain on these chromosomes. Figure 1 shows the distribution of grains over the chromosomes 3, der(3), and der(8), for each of the probes. With pHF12–32, two independent hybridization experiments were done; with pH3E4, three experiments; and with pMS1–37, four experiments. For each probe, the results of the different experiments have been pooled, because different hybridizations with the same probe gave comparable results.

For pH3E4, we found that from a total of 50 grains on the three relevant chromosomes, 29 grains (58%) were on the normal chromosome 3 and 16 grains (32%) on the der(8) chromosome. (The few grains on the der(3) are considered to be part of the background.) There was a clustering of grains at band 3p21, at which 33% of the grains on the normal copy of chromosome 3 and 56% of the grains on the der(8) chromosome were found.

A comparable distribution of grains was observed with pHF12–32. The total number of grains on the three chromosomes was 39, of which 19 (49%) were found on the normal copy of chromosome 3 and 17 (44%) on the der(8) chromosome. On the normal copy of chromosome 3, 58% of all the grains were found in band 3p21. On the der(8) chromosome, 59% of all the grains were in this band.

With pMS1–37, 62 grains were found on either the normal copy of chromosome 3, the der(3) chromosome, or the der(8) chromosome.

Of these grains, 34 (55%) were on the normal copy of chromosome 3, with a clustering of grains in band 3p14 (11 grains, or 32%). On both translocation products, a strong specific hybridization signal seems to be absent. If the three grains found in band 3p14 of the der(3) chromosome should not be considered as background, they only represent a very weak signal.

DISCUSSION

In agreement with our previous study [17], we found 3p21 to be the location of the H3E4 sequence. Probe pMS1–37 has previously been localized by molecular hybridization [18]. Also in accordance with this earlier finding, our in situ hybridization localized the probe to 3p14. The HGM9 listing mentions 3p14--21 as the location of pHF12–32. We could now confine the localization to 3p21.

Both probes pH3E4and pHF12–32 appear to be located distally to the 3p breakpoint in the t(3;8) associated with familial renal cell cancer. Probe pMS1–37 hybridizes very weakly or not at all to either of the translocation chromosomes from the t(3;8), although in the same metaphases a clear signal was obtained at the normal chromosome 3. The absence of a clear signal at both the translocation products is not the result of a deletion of the locus from which this probe originates, because Southern analysis of DNA from a somatic cell hybrid containing the der(3) chromosome as its only human material showed presence of the D3S3 sequence [18]. Chromosomal

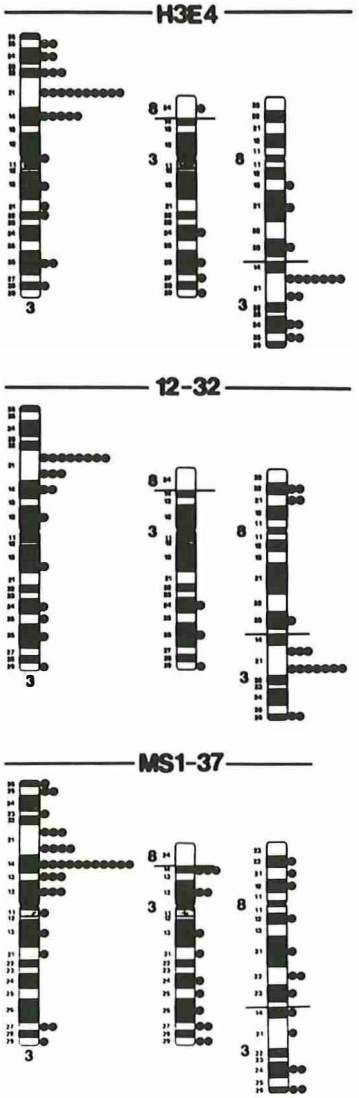

Figure 1 Distribution of grains over the normal chromosome 3 and the two translocation products after in situ hybridization with three 3p probes.

rearrangements may, however, cause a distortion of the higher-order chromosomal structure in the vicinity of the breakpoints involved. If the locus from which a probe originates is very close to a breakpoint, in situ hybridization with that probe may fail because denaturation and/or accessibility of the chromosomal DNA is hampered as a consequence of the structural alteration. The suggestion that a translocation may play a role in changing the accessibility of the chromosomal DNA for hybridization has been mentioned before [19]. Thus, the results obtained with pMS1–37 would mean that D3S3 is very close to.the translocation breakpoint of chromosome 3 in the t(3;8) associated with renal cell cancer. Experiments are now underway to construct

a long-range restriction map around pMS1–37 to establish its distance from the 3p breakpoint.

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3.3 Construction of a long-range restriction map of the heavily methylated genome region around D3S3 using DNA from 5-azacytidine-treated cells

(submitted for publication)

Construction of a long-range restriction map of the heavily methylated genome region around D3S3 using 5-azacytidine treated DNA

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Abstract

Heavy methylation of the relevant chromosomal region can be a major problem in the construction of a long-range restriction map. The region around the locus D3S3 turns out to be such a heavily methylated region. We cultured normal skin fibroblasts in the presence of 5-azacytidine to produce partially demethylated DNA and used this DNA to make a relatively detailed restriction map spanning approximately 1Mb around D3S3. We applied the same conditions to skin fibroblasts from a carrier of the familial t(3;8) associated with renal cell cancer (Cohen et al., New Engl. J. Med. 301 (1979) 592) and analyzed the DNA from these cells. We did not find any indication of the presence of the 3p breakpoint in the mapped region. Culturing cells in the presence of 5-azacytidine is a recommendable approach to analyze heavily methylated genome regions. Although some gross rearrangements may be detected by analyzing long restriction fragments from untreated DNA, smaller rearrangements will escape detection unless a more detailed map can be made, for which partial demethylation of the DNA is an obvious requirement.

Introduction

Cohen et al. (1979) described a family with hereditary RCC and a balanced constitutional translocation t(3;8). By high resolution G-band analysis of prometaphase chromosomes translocation breakpoints were determined at 3p14.2 and 8q24.1 (Wang and Perkins, 1984). Family members with the translocation have an estimated risk of 80% to develop RCC before the age of 60, whereas no RCC has been found in family members with a normal karyotype (Li, 1988). Therefore, it has been suggested that this translocation inactivates one copy of a tumour suppressor gene. A previous study (van der Hout et al., 1991) indicated that the locus D3S3 might be close to the 3p14.2 breakpoint. Thus, D3S3 would be a good starting point to construct a long-range restriction map in search for a tumour suppressor gene. Aberrant fragments in DNA from a translocation carrier would

pinpoint the location of the breakpoint. Mapping, however, turned out to be difficult because the restriction fragments recognized by the probe defining the D3S3 locus were excessively large. This has been noticed earlier (Gemmill et al., 1986) and may well be caused by heavy methylation of the chromosomal region around D3S3. The recognition sequences of most rare cutter restriction enzymes contain one or more CpG dinucleotides and are methylation sensitive. When the cytidine in such a CpG is methylated there will be no cleavage of the sequence. Therefore, we decided to treat the cells from which we wanted to isolate DNA for mapping studies with the demethylating agent 5-azacytidine (5-azaC). Thus, we hoped to make more sites accessible for cleavage by rare cutter restriction enzymes (Dobkin et al., 1987, Heard and Friend, 1990) and thereby to create smaller restriction fragments.

Materials and methods

Normal human skin fibroblasts or skin fibroblasts from a carrier of a balanced translocation t(3;8)(p14.2;q24.1) (fibroblasts 2954) were cultured in Ham's F12 medium supplemented with 15% fetal calf serum. For 5-azaC treatment cells were grown in the same medium containing 5-azaC (Sigma). Throughout this study we used a concentration of 2.5 μM as proposed by Dobkin et al. (1987). Growth of the fibroblasts was retarded on the 5-azaC medium. On normal medium the population doubling time was 2-3 days, whereas for 5-azaC-treated cultures it was 9-10 days. After three weeks of culture cells were harvested, because keeping them longer on 5-azaC medium led to a rapid deterioration of their condition. Scince it has been suggested that 5-azaC may not be stable in solution for more than 3 weeks (Dobkin et al., 1987) we kept it as a 2000x stock solution at 4°C for no longer than this period. Medium was changed 3 times a week and 5-azaC was added from the stock solution just before the medium was given to the cells.

Cells were harvested, washed in 0.144 M NaCl, 0.012 M Na-phosphate, pH 7 (PBS), counted, embedded in 0.5% lmp-agarose in PBS at a final concentration of $7.5x10^6$ cells/ml, and formed into agarose plugs of 100 μl. Plugs were incubated

in 0.5 M EDTA (pH8), 1% lauroyl sarcosine, and 1 mg/ml proteinase K at 50°C for 40 h. After that, plugs were rinsed in 10 mM Tris/Cl, 1 mM EDTA, pH 8 (TE8), incubated in TE8 + 40μg/ml phenyl methyl sulfonyl fluoride at 50°C for 30 min and again rinsed extensively in TE8. Plugs were stored in TE8 at 4°C.

Before digestion with a restriction enzyme plugs were incubated in the appropriate restriction enzyme buffer for 2 times 30 minutes. Digestion was carried out with 10-15 U/μg DNA for 5 h at the temperature recommended by the supplier (Pharmacia). Per lane 0.5 plug was loaded (approximately 3 μg DNA). Gels were run on a Pulsaphor apparatus (LKB-Pharmacia) using the hexagonal electrode, at 10°C, in 0.5 x TBE buffer (45mM Tris base, 45mM boric acid, 1mM EDTA, pH8). Exact electrophoresis conditions are mentioned in the legends to the Figures. Probe labelling, Southern blotting, and hybridization were carried out as described elsewhere (van der Hout et al., 1991) with the modification that gels were incubated in 0.25 N HCl for 15 min, before blotting. Blotting was overnight at 4°C. Blots were hybridized with purified insert from probe PMS1-37 defining the locus D3S3 (Williamson et al., 1991).

Results

Digests of normal control fibroblast DNA were resolved using an electrophoresis program which separates fragments up to approximately 1600 kb. Probe pMS1-37 detected NotI, MluI, and NruI fragments which appeared to stay in the compression zone (Fig.1, lanes 5 and 7, Fig.3, lane 2). In SfiI digests bands of 1500, 735, and 630 kb were detected (Fig.1, lane 1). Digests with other enzymes (SalI, SmaI, SacI, ClaI) which give on average smaller bands than enzymes like NotI, MluI, and NruI all showed multiple bands, also after prolonged digestion (e.g. ClaI digest in Fig.1, lane 10). Using another electrophoresis program which separates fragments up to approximately 6000 kb, two fragments of approximately 3000 kb and 4600 kb were detected in MluI digests (Fig.2, lane 2). Hybridizing NotI and NruI fragments, however, still remained unresolved under this conditions (results not shown).

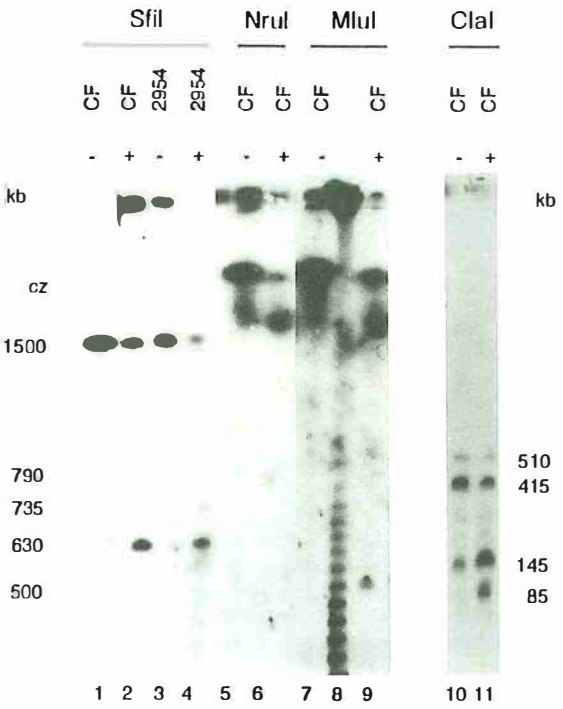

Fig.1. Fragments detected by pMS1-37 in SfiI, NruI, MluI and ClaI digests of untreated (-) and 5-azaC treated (+) skin fibroblast DNA. CF=control fibroblasts, 2954=carrier of t(3;8). Lane 8 contains lambda DNA ladder (Pharmacia). cz=compression zone. Digests were resolved on a 1% agarose gel at 120 V. Pulse times were ramped linearly from 20 s to 2 min over 15 h, hold constant at 2 min for 34 h, and then ramped from 2 min to 10 min over 16 h.

On the contrary, in DNA from 5-azaC-treated fibroblasts separated by the first program pMS1-37 detected a 790 kb NruI fragment (Fig.1, lane 6) and a 500 kb MluI fragment (Fig.1, lane 9). On the autoradiogram of the SfiI digest a shift in band intensities was observed towards the smallest fragment (Fig.1, lane 2). The same holds for the ClaI digest (Fig.1, lane 11). In the NotI digest of 5-azaC treated DNA still only a signal in the compression zone could be detected (results not shown). In Fig.3 hybridization results of pMS1-37 to single and double digests of 5-azaC treated DNA with NotI, MluI, NruI, and SfiI are shown. Fragment lengths are listed in Table 1. The restriction map derived from this data covers 915 kb around the locus D3S3 (Fig.4). When DNA from fibroblasts from a carrier of the familial t(3;8) was compared to control fibroblast DNA similar patterns were observed, except for the absence of one very long MluI fragment (Fig.2). No aberrant fragments were detected, however, neither in 5-azaC treated DNA nor in untreated DNA (e.g. Fig.1 lanes 1-4, Fig.2) .

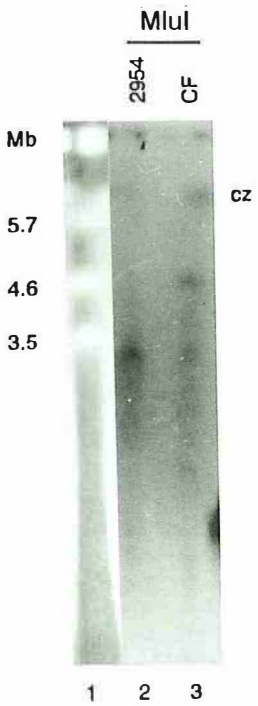

Fig.2. Fragments detected by pMS1-37 in MluI digests of control skin fibroblast DNA (CF, lane 3) and skin fibroblast DNA from a carrier of the t(3;8) (2954, lane 2). Lane 1 contains S. pombe size markers (Biorad). cz=compression zone. Digests were resolved on a 0.5% agarose gel at 90 V. Pulse times were ramped linearly from 15 min to 60 min over 230 h.

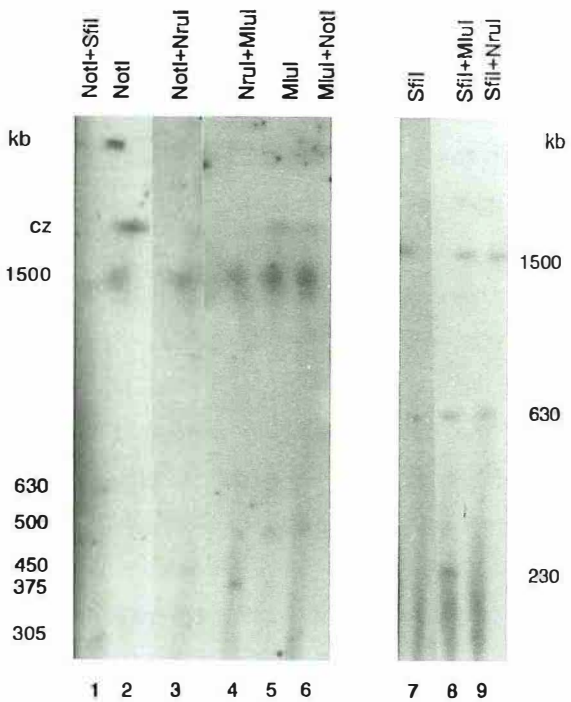

Fig.3. Fragments detected by pMS1-37 in single and double digests of 5-azaC treated control skin fibroblast DNA. cz=compression zone. Electrophoresis conditions were as described in the legend to Fig.1

	NotI	SfiI	MluI	NruI
NotI	-	305	500	450
SfiI		630[1]	230	630
MluI			500	375
NruI				790

Table 1. Restriction fragments detected by pMS1-37 in digests of DNA from 5-azaC-treated skin fibroblasts.
[1]For SfiI only the smallest fragment is listed.

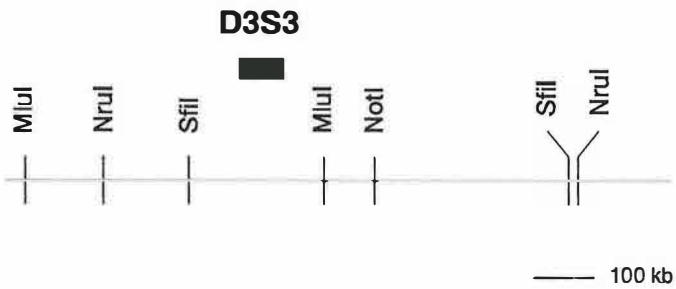

Fig.4. Long-range restriction map around the locus D3S3.

Discussion

The construction of a long-range restriction map of the region around the locus D3S3 was hampered by the excessively long restriction fragments that were detected in our fibroblast DNA by the probe defining this locus. Not only were these fragments with a size of several megabases difficult to separate, but even if they could be separated they were difficult to measure because of the lack of marker bands in this size class. With some enzymes (SfiI, SmaI, ClaI, SalI) smaller fragments were found, but even after very exhaustive digestion multiple hybridizing fragments were still seen. The presence of relatively many hybridizing fragments would make interpretation of double digests very complicated. Thus, since these enzymes, therefore, did not suit well our mapping

purposes, we tried to demethylate the DNA by growing the cells in a medium containing 5-azaC. Indeed, in DNA from treated cells we found substantially shorter fragments, which indicates that heavy methylation, and not some other feature like a very low AT content of the region, is the cause of the paucity of available restriction sites around D3S3. As can be seen in Figs. 1 and 3 the demethylation of certain sites is only partial. There is still a strong hybridization signal in the compression zone, but additional shorter hybridizing fragments appear. Hybridization with a probe from a different region resulted in a single hybridizing fragment, indicating that there is no question of partial digestion in general (results not shown). Further demethylation could not be obtained by prolonged treatment of the cells with 5-azaC, since that caused death of the culture. The 5-azaC treatment gave reproducible patterns of hybridizing restriction fragments with DNA isolations from a number of similar cultures (results not shown).

Based upon the length of the fragments detected in the various single and double digests of the 5-azaC treated DNA a map was constructed containing the restriction endonuclease sites involved. This map spans 915 kb around D3S3. As no aberrant bands were found in DNA of the carrier of the t(3;8), it is unlikely that the translocation breakpoint lies within these 915 kb. In a MluI digest of control DNA 2 fragments of 4.6 and 3 Mb were detected, whereas in the DNA from the t(3;8) carrier only the 3 Mb fragment was detected (Fig.2). Although it cannot be ruled out that the translocation breakpoint is located just outside the 3 Mb MluI fragment and that an aberrant breakpoint-spanning fragment only slightly larger than 3 Mb is generated which would not be detected as a separate band, the presence of the longer band in the control DNA may well be caused by partial digestion, as may its absence in the t(3;8) DNA by a possible less quality, i.e. lack of very long fragments.

To find the location of the breakpoint we plan to extend the map by using probes derived from a 3p14 specific microdissection library.

The locus D3S3 is included in a region homozygously deleted from the small cell lung cancer cell line U2020 (Rabbitts et al., 1990). Probes missing from the U2020 cell line, including D3S3, have been mapped either to 3p13-14.2 using a panel of somatic cell hybrids (Drabkin et al., 1992) or to 3p12 using in situ

ıybridization (Latif et al., 1992). The latter finding is in contrast to earlier in situ hybridization studies that mapped D3S3 to 3p13-3p14.2 (Albertson et al., 1989) or 3p14 (van der Hout et al., 1991). This raises the question of the exact location of D3S3. If the 3p12 assignment would be correct, D3S3 would be far away from the t(3;8) breakpoint.

One of the above-mentioned studies (Drabkin et al., 1992) presented a tentative map of the D3S3 region using data from pulsed field gel analyses of different cell lines showing cell-line specific variation. The map of approximately twice the size of our map was designed only to give a probable order and an estimate of distances for some markers in the region. It contained several inconsistencies as stated by the authors themselves. Precise positions for the fragments could not be given because double digestions had not been carried out. An MluI fragment of 500 kb, an NruI fragment of 790 kb, and SfiI fragments of 1500, 735, and 630 kb are results in common in both the study of Drabkin et al. (1992) and ours. The method of using partially demethylated DNA enabled us to include data from double digests and, thereby, to make a more detailed map.

Acknowledgements

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3.4 Characterization of a 3p14 microdissection library and its use in the construction of a long-range restriction map of the 3p region involved in a t(3;8)(p14.2;q24.1) associated with hereditary renal cell carcinoma

Characterization of a 3p14 microdissection library and its use in the construction of a long-range restriction map of the 3p region involved in a t(3;8)(p14.2;q24.1) associated with hereditary renal cell carcinoma

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Abstract

We characterized a 3p14-specific microdissection library to obtain markers for analyzing the breakpoint in a t(3;8)(p14.2;q24.1) associated with familial renal cell carcinoma. Unique sequence clones from both sides of the t(3;8) breakpoint were used in a pulsed-field gel electrophoresis analysis of the 3p14 area. Physical linkage between several clones was established. A segment of 4.6 Mb in 3p14.1-p14.2 was mapped in more detail. This map may serve as a starting-point to bridge the translocation. Moreover, one of the microclones could be used to extend an existing long-range map in 3p21; a group of four clones appeared to define the end of the 3p segment in the hybrid cell line DIS2.6; and 5 new restriction fragment length polymorphisms were detected by microdissection clones.

Introduction

A straightforward method to get probes from a specific chromosome region is dissection of the region from metaphase chromosomes using a micromanipulator, and subsequent cloning of DNA from the dissected fragments into a suitable vector. Thus, a region-specific library can be constructed. This approach has first been applied to Drosophila polytenic chromosomes (Scalenghe et al., 1981). The technique was adapted for mammalian chromosomes by Rohme et al. (1984). A procedure including a very brief fixation of metaphase chromosomes, G-banding of chromosomes, and amplification of the microdissected material by PCR (Lüdecke et al., 1989,

1990, Senger et al., 1990) has been used to construct libraries containing 5000-20,000 clones from the following chromosome regions: 5q11-12 (Buiting et al., 1990), 11p13 (Davis et al., 1990), Xq27-28 (Mackinnon et al., 1990), 22q12-13.1 (Fiedler et al., 1991), 5q21.3-22 and 5q22 (Hampton et al., 1991), 8q24.1 (Lüdecke et al., 1991), 11p15.5 (Newsham et al., 1991), and 3p14 (this paper).

We were interested in the 3p breakpoint of a balanced constitutional translocation (3;8)(p14.2;q24.1) which seems to predispose to renal cell carcinoma (RCC) in a family described by Cohen et al. (1979). In order to physically localize this breakpoint we characterized a microdissection library of 3p14 and used unique sequence clones to construct a long-range restriction map of this chromosome region which we wanted to compare to a similar map from DNA of a carrier of the t(3;8). Restriction fragments spanning the breakpoint would be recognized (1) by an aberrant pattern in DNA of the translocation carrier upon hybridization with a clone recognizing such fragments, and (2) by similar patterns in control DNA upon hybridization of clones located distal and proximal to the breakpoint.

Materials and methods

Cell lines

The following cell lines were used for localization of clones: human/hamster hybrid cell lines UCTP 2A3 (containing an intact chromosome 3 as its only human material), TL9542/UC/12-8 (containing as its only identifiable human material the der(3)t(3;8)(p14.2;q24.1) from the familial t(3;8) predisposing for RCC), 3;8/4-1 (containing the der(8)t(3;8)(p14.2;q24.1) from the familial t(3;8) in the absence of the der(3) and the normal human chromosomes 3 and 8) (Gerber et al., 1988), DIS2.6 (containing 3p21-24, distal of D3S2, as its only human chromosome 3 material) (Carritt et al., 1992), small cell lung cancer cell line U2020 with a submicroscopic homozygous deletion in 3p13-p14.2 or 3p12 (Rabbitts et al., 1990; Drabkin et al., 1992; Latif et al., 1992), and a somatic cell deletion mapping panel containing terminal 3p deletions with different breakpoints in the 3p11-p22 region (Wang et al., 1992). Moreover we used skin fibroblasts 2954 from a carrier

of the familial t(3;8) containing the der(3), der(8), and a normal copy of chromosome 3 (Cohen et al., 1979) and control skin fibroblasts from a normal individual.

Microdissection and microcloning

Microdissection and microcloning have been described in detail elsewhere (Lüdecke et al., 1989, 1990, Senger et al., 1990). Shortly, chromosome band 3p14 was dissected from 20 G-banded metaphase chromosomes of normal human lymphocytes, DNA was extracted, digested with RsaI, and ligated into a specially engineered pUC plasmid ("SmaI vector"). Inserts were amplified by PCR and this primary amplification product was ligated into pUC13. From this ligation mixture 1 µl was used to transform competent E.coli DH5α cells (BRL). After recovery all cells were plated.

Fluorescent in situ hybridization

For use as a probe in fluorescent in situ hybridization 1 µl of the primary amplification product was subjected to another 25 cycles of PCR using the universal M13/PUC sequencing and reverse-sequencing primers. In a 30 µl reaction we used 0.125 U SuperTaq polymerase (SphaeroQ) in the buffer provided by the supplier, supplemented to contain 1.8 Mm $MgCl_2$, 10 ng/µl of each of the primers, and 200 µM each of DATP, DGTP, DCTP, and DTTP in a 30 µl reaction. Each cycle consisted of denaturation at 92°C for 30 s, annealing at 45°C for 30 s, and primer extension at 72°C for 60 s. The first denaturation was at 93°C for 3 min and the final primer extension at 72°C for 4 min. The PCR product was purified and concentrated using the Band Prep kit (Pharmacia). This material was biotinylated and hybridized to metaphase chromosomes from fibroblasts 2954 essentially according to Kievits et al. (1990). For competitor DNA we used cotI DNA (BRL) in 50x excess over the labelled probe. Per slide 250 ng probe DNA was applied. The hybridization was at 37°C for 3 days.

Analysis of cloned inserts and subchromosomal localization

Plasmid DNA was extracted from 3 ml bacterial cultures using the alkaline lysis method (Sambrook et al. 1989). Inserts were amplified by PCR using the M13/pUC universal sequencing and reverse sequencing primers as described in the previous paragraph. Amplified insert material was labelled by primer extension labelling using α^{32}P-dCTP and the same primers as used for the PCR. Amplified insert material was hybridized to filters containing EcoRI digested DNA from human placenta, rat liver (to screen for possible evolutionary conserved sequences), and hybrid cell lines UCTP 2A3, TL9542/UC/12-8, 3;8/4-1, and DIS2.6. Unique sequence clones located proximal to the t(3;8) breakpoint (clones which showed single copy hybridization signals on placenta DNA, UCTP 2A3, and TL9542/UC2/12-8) were also hybridized to filters containing EcoRI-digested DNA from small cell lung cancer cell line U2020 and genomic control DNA. Hybridized filters were exposed to X-ray films at -70°C for 1-5 days. A number of unique sequence clones was localized more precisely using a panel of somatic cell hybrids containing terminal 3p deletions with different breakpoints in the 3p11-p21 region.

Search for restriction fragment length polymorphisms

Unique sequence clones with a positive localization on chromosome 3 were used to search for restriction fragment length polymorphisms (RFLP). They were hybridized to filters containing DNA from UCTP 2A3 and a mixture of 5 human placenta DNAs, digested with DraI, PstI, BamHI, BglII, TaqI, HindIII, MspI, or EcoRI. When a clone detected an additional band in the lane with the mixture of placenta DNAs, the presence of an RFLP was verified and the frequency of the different alleles was established by hybridizing the clone to a filter containing 20 placenta DNAs digested with the relevant restriction enzyme.

Long-range restriction mapping

Preparation of DNA in agarose plugs, restriction enzyme digestion, and pulsed-field gel electrophoresis were as described in section 3.3. In case of double digestions

agarose plugs were incubated with the first enzyme for 4-5 h, washed with 10 mM Tris, 1 mM EDTA, pH 8, incubated at 4°C overnight in the restriction buffer recommended for the second enzyme, and then incubated with the second enzyme for 4-5 h. Unique sequence clones were hybridized to pulsed-field filters containing DNA from normal control fibroblasts and fibroblasts 2954 digested with SfiI, MluI, NruI, NotI, SacII, and combinations of two of these enzymes. Exposure to X-ray film of pulsed-field filters hybridized with microclones was 10 days at -70°C. To confirm that two clones recognized identical fragments such clones were sequentially hybridized to the same filter.

Results

Microdissection and microcloning

A library containing by estimation 20,000 clones was constructed from the dissected 3p14 material. Transformation of competent cells with 1 µl of amplified material cloned into pUC13 yielded 650 recombinant colonies which were stored individually.

Fluorescent in situ hybridization

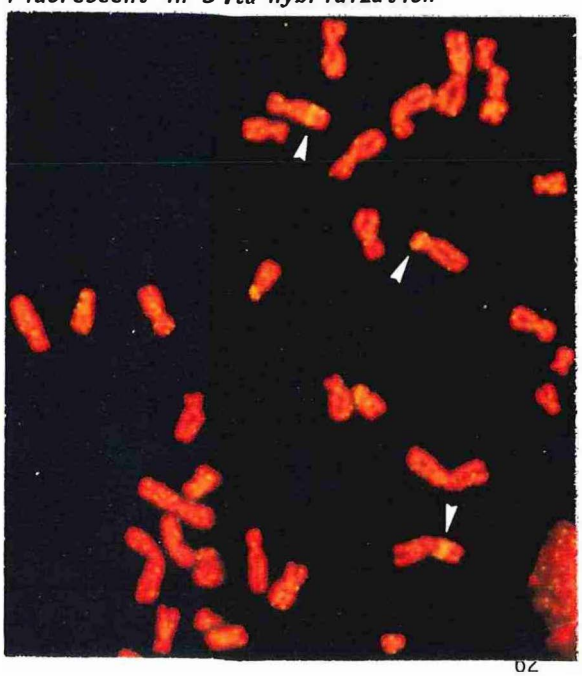

Fig.1 In situ hybridization on metaphase chromosomes from fibroblasts of a carrier of the t(3;8) using the 3p14 microdissection library as a probe. Arrows indicate the normal chromosome 3, the der(3), and the der(8).

The primary amplification product of the library was subjected to another PCR to yield enough material to be used as a probe for in situ hybridization. The amplified material was labelled and hybridized to metaphase chromosomes from a carrier of the familial t(3;8). As is shown in Fig.1, three signals were present: one at 3p14 on the normal chromosome 3 homologue, and one on each of the translocation products, indicating that the library contains sequences from both sides of the breakpoint.

Analysis of individual clones and subchromosomal localization

A summary of the analysis is shown in Table 1. Out of a total of 88 clones analyzed 38 unique sequence clones originated from chromosome 3. Five different clones occurred in 2 copies and 1 clone in 3 copies. Thus, 31 clones turned out to represent independent unique sequence clones (i.e. 35% of the clones analyzed).

Table 1. *Characterization of microclones*

	n=
analyzed clones	88 (100%)
no signal	30 (34%)
repetitive	11 (13%)
single-copy	47 (53%)
not located on chromosome 3	9 (10%)
independent single-copy clones, located on chromosome 3	31 (35%)

The average length of PCR amplified products from these clones was 225 bp (ranging from 160-460 bp). Since these PCR products contain 119 bp of primer annealing sites and other vector sequence the average insert length for these microclones was 105 bp. Based on hybridization to hybrid cell lines 19 clones (61%) were localized distal to the 3p14.2 breakpoint and 12 (39%) proximal. Two of the distal clones (nrs. 2 and 3) hybridized to the hybrid cell line DIS2.6, implicating a location in 3p21-24. All proximal clones hybridized to DNA of the U2020 cell line. Therefore, they were not located in the region deleted from this cell line.

Table 2. *Localization of, and restriction fragments recognized by, unique sequence microdissection clones*

		RESTRICTION FRAGMENTS (kb)				LOCALIZATION	
clone#	lab-code	NotI	MluI	NruI	SacII	t(3;8)	s.c.h.p[1].
1	2C6		***1500***			d	
2	W14	630	435	490		d	
3	LB1.9	780	***1500***	780		d	
4	W2.8	780	***1500***	780		d	3p21.1-24.2
5	2C4	780	***1500***	780	720 660	d	
6	2A11	780		780	720 660	d	
7	2B11	***1500***	***1500***			d	3p21.1
8	1A3	***1500***				d	3p21.1
9	LB8	***150*** 320	420			d	3p21.1
10	W20		600			d	3p14.3-21.1
11	LB3.6	2500				d	3p14.3-21.1
12	W19	2500	720		1500	d	3p14.3-21.1
13	LB4.1	2500	720		1500	d	3p14.3-21.1
14	LB5.6	>1000	>1000			d	3p14.2-14.3
15	LB2.2	>900	>2000	>2000	2500	d	3p14.2
16	1A2	>1000	>1000			d	3p14.2
17	LB2.3	>2000	>2000	>1600	>1600	d	3p14.2
18	2A5	2300	2500	3000	580	p	3p14.1-14.2
19	2H1	2300	>2000	1750	220	p	3p14.1-14.2
20	2B6	680	>2000	1750	2300	p	3p14.1-14.2
21	W2.9	680	>1500	1750	2300	p	3p14.1-14.2
22	W3.7	1850	>1600	1050		p	3p14.1-14.2
23	W3.2	2000			720	p	3p14.1-14.2
24	2A9	2500	2000		300	p	
25	2B1	640				p	
26	2B7	>2000	1600	>2000	390	p	
27	2C5	460	510	610	390	p	
28	2C9	610 220				p	

Boxes surround identical fragments, interrupted lines indicate fragments were detected on separate filters. Lengths of fragments in italics are by approximation because these were detected in a region of a gel where separation was not optimal. The line between clones 19 and 20 indicate the location of the t(3;8) breakpoint. [1] *localization using somatic cell hybrid panel* [2] *d: distal to t(3;8) breakpoint, p: proximal to breakpoint.*

In Table 2 a more precise localization can be found for some of the clones as established with the use of a panel of somatic cell hybrids containing terminal 3p deletions with different breakpoints in the 3p11-p21 region. No clones hybridized to rat liver DNA, nor were apparent hamster-specific bands detected in DNA of the hybrid cell lines. Therefore, there were no indications for clones containing evolutionary conserved sequences.

Restriction fragment length polymorphisms

Amongst the 31 unique sequence clones 3 clones detected 1 RFLP each, 1 clone detected 2 RFLPs. Table 3 gives the specifications of these RFLPs.

Table 3. *Restriction fragment length polymorphisms*

clone	localization	enzyme	alleles	frequency
16 (1A2)	3p14.2	TaqI	4.3 kb	0.94
			3.6 kb	0.06
21 (W2.9)	3p14.1-p14.2	HindIII	11.5 kb	0.43
			8.8 kb	0.57
23 (W3.2)	3p14.1-p14.2	MspI	5.2 kb	0.45
			4.6 kb	0.24
			1.5 kb	0.31
22 (W3.7)	3p14.1-p14.2	MspI	7.3 kb	0.40
			6.2 kb	0.60
		TaqI	4.3 kb	0.50
			4.1 kb	0.50

Long range restriction mapping

Results from hybridizations of unique sequence clones to pulsed-field filters are listed in Table 2. No differences were observed between bands detected in DNA from control fibroblasts or from fibroblasts 2954, which are from a carrier of the t(3;8). SfiI fragments were almost all <400 kb and therefore of limited use in establishing physical linkage between clones. For that reason they are not in the

Table. Some physical linkages are suggested by the data in Table 2. It is likely that clone nrs.12 and 13, both located in 3p14.3-p21.1 are located on the same NotI, MluI, and SacII fragments. Probably also clone nr.11 is on the same NotI fragment. The SacII fragments recognized by clones nrs. 26 and 27, respectively, are most likely two fragments that incidentally have the same length, since NotI, MluI, and NruI fragments recognized by these clones are nonidentical. Clones nrs. 3, 4, 5, and 6 all recognized the same NotI and NruI fragments. Although we have not yet identified an MluI fragment with clone nr.6 this clone also recognizes the same SacII and SfiI (not in Table 2) fragments as does clone nr.5. Therefore, clones nrs. 3, 4, 5, and 6 are considered to belong to one linkage group. Double digests did not add any information on the order of the clones within this group.

Four clones, nrs.18, 19, 20, and 21 in Table 2, all located in 3p14.1-p14.2, appeared to recognize overlapping restriction fragments. These clones were hybridized to filters containing double digests of control fibroblast DNA. Fragment lengths are listed in Table 4. We constructed a 4.6 Mb restriction map containing these four clones (Fig.2).

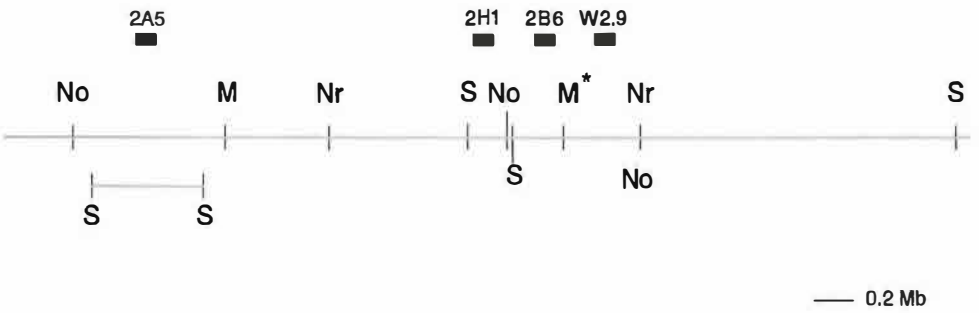

Fig.2 Long-range restriction map of 3p14.1-p14.2. No: NotI, M: MluI, Nr: NruI, S: SacII. The location of the MluI site marked (*) between 2B6 and W2.9 and of the SacII fragment recognized by 2A5 are not fully established yet.

Table 4. *Restriction fragments recognized by microdissection clones in double digests*

clone:	18 (2A5)	19 (2H1)	20 (2B6)	21 (W2.9)
NotIxMluI	800	>1000	350	420
NotIxNruI	>1000	930	680	680
NotIxSacII	580	200	610	610
MluIxNruI	>1000	>1000	>1000	420
MluIxSacII	n.d.	n.d.	315	>1000
NruIxSacII	n.d.	n.d.	610	610

restriction fragments in kb; n.d.=not done

Discussion

Based on the localization of the relatively small number of individual clones analyzed, about 60% of the 3p14 microdissection library is derived from the chromosome segment distal to the t(3;8) breakpoint and 40% from the proximal side. The most distally localized clones, nr.4 and three clones physically linked to it, mapped to 3p21.1-p24.2. The most proximal clones are most likely still distal to the region deleted in the cell line U2020, as none of the proximal clones maps in this large (4-7 Mb; Latif et al., 1992) deletion. Dissection of a segment somewhat broader than just band 3p14 from one or more of the 20 dissected chromosomes can easily explain the presence of clones from more distal or proximal bands.

The average insert length in the 3p14 library is smaller than in comparable libraries (105 bp vs. 150-220 bp). The percentage of clones that did not hybridize is similar, the percentage of repetitive clones is lower (13% vs. 20-52%). All libraries, like this one, have few redundant clones (Lüdecke et al., 1990). Although the clones from this microdissection library are rather short, they appeared to be well usable as probes for hybridization to pulsed-field filters.

From clones nrs. 3, 4, 5, and 6 that appeared to be physically linked (Table 2), nr.3 is contained in DIS2.6, the other three clones are not. Clone nr.4 and

consequently the whole linkage group were localized to 3p21.1-p24.2 according to the somatic cell hybrid panel. DIS2.6 contains the segment 3p21-p24 (Carritt et al., 1992). Our results indicate that one end of the 3p segment in DIS2.6 is located between clone 3 and one of the other three clones in this group, or that we have discovered a discontinuity in this DIS2.6 fragment.

Clone nr.2 recognized the same NotI and MluI fragment as a probe from the D8 locus, located in distal 3p21 (Carritt et al., 1992). With fragments from a cosmid (cW14) isolated by screening a cosmid library with clone nr.2 we were able to confirm the position of the next NruI site in an existing map of this region (Carritt et al., 1992) and to add the next NotI site (approx. 90 kb further) directed away from APEH (located 140 kb from D8 in the other direction).

We made a long-range restriction map spanning 4.6 Mb with 4 clones located in 3p14.1-p14.2, proximal to the t(3;8) breakpoint (Fig.2). The orientation of the map with respect to the t(3;8) breakpoint could not be inferred from the collected data. Yamakawa et al. (1992) also made a pulsed-field analysis of the 3p14 region. They used 11 markers distal to the t(3;8) breakpoint and 11 from the proximal side, all localized by in situ hybridization to 3p14.1-p21.1 (Takahashi et al., 1992). Several markers could be linked on identical restriction fragments. No physical linkage was found between markers proximal and distal to the t(3;8) breakpoint, nor were aberrant fragments detected in DNA from a carrier of the t(3;8). There are apparently no identical NotI, MluI, or NruI fragments in their results and in our results as shown in Table 2. The region 3p14-p21 comprises approx. 20 Mb. Therefore, the absence of an overlap between our results and those of Yamakawa et al. (1992) is not unaccountable.

Fragments detected by the microdissection clones (Table 2) apparently also did not show any overlap with two maps constructed around D3S3 by us (section 3.3) and by Drabkin et al. (1992). The D3S3 region (contained within the U2020 deletion, Rabbitts et al., 1990) is probably not covered by this microdissection library. Gemmill et al. (1991) constructed a map covering approximately 2 Mb, which links 5 markers, of which D3S2 is the middle one, to a translocation breakpoint (associated with Greig cephalopolysyndactyly syndrome) located in 3p21.1. Fragments detected by clones nrs. 3, 4, 5, and 6 might be identical to fragments detected by

robe 199H4 of Gemmill et al. (1991), which is located within 150 kb proximal of he Greig breakpoint.

Our results indicate that the t(3;8) breakpoint is located between one of the clones in 3p14.2 and one of the clones in 3p14.1-p14.2 (Table 2), excluding nrs. 9 and 20 which have an internal location in the map of 3p14.1-p14.2 (Fig. 2).

Acknowledgements

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CHAPTER 4

Somatic cell genetic criteria in the classification of renal cell tumours

4.1 Introduction

Renal cell tumours are histologically and clinically a very heterogeneous group. In order to relate a specific course to a specific subtype of RCC it is important to have a good definition of the different subtypes. Since genetic events are at the very beginning of the process which turns somatic cells into tumour cells and since differences at a DNA level will underlie the different routes of development leading to different subtypes of a tumour, correlation of genetic characteristics with cytological expression may contribute significantly to such a definition. We studied whether allele loss on chromosome 3 or specific sets of chromosome abnormalities could be used as classifying criteria. The former is discussed in section 4.2, the latter in section 4.3.

From our study of LOH on 3p we learned that in about half of the tumours no LOH could be detected. We wondered whether LOH on 3p was restricted to a specific histopathological subtype of RCC. and, therefore, we compared loss of heterozygosity data with different RCC classifications. When we applied the classification as proposed by Thoenes et al. (1986), which is based upon the types of renal cells from which the different tumours originate, tumours with LOH were only found in the clear cell category of this classification. This might indicate that either LOH on 3p is restricted to a subset of RCC (clear cell tumours according to Thoenes et al., 1986), or LOH in non-clear cell tumours is much more local and cannot be detected by the set of markers we used in the work described in section 4.2. Kovacs et al. (1992), reported allelic losses on 3p in 5 of 9

informative cases of chromophobic RCC. For both oncocytomas and chromophobic cell carcinomas similar abnormalities of mitochondrial DNA have been reported (Welter et al., 1989; Kovacs et al, 1992). The meaning of these abnormalities that have not been found in other subtypes of RCC remains unclear.

Molecular and cytogenetic genome analyses need to complement each other. Abnormalities occurring in only a minority of cells from a certain tumour specimen can only be detected by cytogenetic analysis. On the other hand, events like mitotic recombination or loss of a homologue followed by duplication of the remaining copy of a chromosome are microscopically invisible but can be detected by DNA analysis (see sections 4.2 and 4.3).

In section 4.3 cytogenetic characteristics are also related to tumour stage. Specific combinations of chromosome abnormalities were only found in grade 2 and 3 tumours and may, therefore, reflect tumour progression within subtypes of RCC. Such a correlation has also been suggested for the accumulation of allele losses, c.f. discussion section 2.1. Thus, genetic analysis might have a prognostic value in addition to a diagnostic significance.

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4.2 Loss of heterozygosity at the short arm of chromosome 3 in renal-cell cancer correlates with the cytological tumour type

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LOSS OF HETEROZYGOSITY AT THE SHORT ARM OF CHROMOSOME 3 IN RENAL-CELL CANCER CORRELATES WITH THE CYTOLOGICAL TUMOUR TYPE

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A majority of renal-cell tumours retain heterozygosity at the short arm of chromosome 3. To investigate possible histopathological differences between tumours with and without such losses, we compared loss of heterozygosity data from 51 tumours with 1 histological and 2 different cytological classifications of renal-cell tumour. Using the cytological classification of Thoenes *et al.*, we only found tumours with loss of heterozygosity in these authors' clear-cell category. Possibly, only these tumours arise by a mechanism of double loss of a tumour-suppressor gene on 3p, non-clear-cell renal tumours having a different genetic background. Alternatively, deletions may occur in all subtypes, in which case those subtypes in which no LOH is found may also contain deletions too small to be detected with the set of 3p probes we used. A cytogenetic analysis was carried out on 30 of the tumours. Results of molecular and microscopic cytogenetic analyses did not seem to be in agreement in 12 cases. In 6 of these we found allelic losses in tumours showing morphologically normal copies of chromosome 3. Mitotic recombination or loss of one chromosome 3 homologue followed by duplication of the remaining homologue is a likely explanation. The other 6 cases showed microscopic abnormalities of chromosome 3 which were not reflected, or only partly reflected, as allelic losses. These discrepancies are caused either by the limitations of microscopic analysis in exactly determining a breakpoint or tracing a translocated part of a chromosome, or by the failure of molecular analysis to demonstrate LOH if this occurs in only a minority of cells.

In cytogenetic studies of renal-cell carcinoma (RCC), deletions and translocations involving the short arm of chromosome 3 are frequently found (Yoshida *et al.*, 1986; de Jong *et al.*, 1988; Kovacs and Frisch, 1989; Walter *et al.*, 1989, Presti *et al.*, 1991). These rearrangements often result in the heterozygous loss of the distal part of 3p, with breakpoints in the region 3p11-21. According to the theory of Knudson (1985), this region may harbour a tumour-suppressor gene. Functional loss of both alleles of such a gene is supposed to be the first step in the development of RCC. By studying DNA from matched pairs of RCC-tumour/normal kidney tissue for loss of heterozygosity (LOH) a ready analysis of 3p deletions can be made (Zbar *et al.*, 1987; Kovacs *et al.*, 1988; van der Hout *et al.*, 1988) and the location of the presumed suppressor gene can be defined more precisely (van der Hout *et al.*, 1991; Yamakawa *et al.*, 1991). Cytogenetic and molecular studies published so far could not reveal loss of 3p sequences in every renal-cell tumour. This may be a consequence of the occurrence of rather small deletions, too small to be detected by the set of probes currently available. Since, however, RCC is claimed to be heterogeneous from a histological or cytological point of view, an alternative explanation may be that the presence or absence of 3p deletions is reflecting this heterogeneity. We therefore investigated whether a classification of renal-cell tumours based on a DNA analysis of chromosome 3 would be compatible with any existing classification based on cytological or histological criteria.

MATERIAL AND METHODS

In a previous study (van der Hout *et al.*, 1991) we investigated loss of heterozygosity at chromosome 3 in 41 renal-cell tumours. Of these, 7 could not be evaluated histologically and cytologically. For the present study, 17 further cases have been added. These were tested for LOH using the same polymorphic probes as in the previous study with the addition of pEFD145.1 identifying the locus D3S32 in 3p21 (Williamson *et al.*, 1991). Together, these probes recognize 15 RFLPs of 3p and 3 RFLPs of 3q. Histological and cytological features of all 51 tumours were scored without knowledge of the results of the DNA analyses. The tumours were grouped according to 1 histological and 2 different cytological classifications. They were histologically classified as solid, papillary, tubular, or with mixed patterns. The first cytological classification (A in Table I) is based upon common criteria as recommended by the WHO (Mostofi, 1981) and up to now generally applied (Ogawa *et al.*, 1991; Presti *et al.*, 1991; Anglard *et al.*, 1991, 1992; Yamakawa *et al.*, 1991). This classification distinguishes between tumours of clear-cell, granular-cell, spindle-cell, oncocytic-cell and mixed-cell types. The second classification (B) is that proposed by Thoenes *et al.* (1986) who distinguished between clear-cell carcinoma, chromophilic-cell carcinoma, chromophobic-cell carcinoma, oncocytoma, and the cell type of carcinoma of Bellini's duct. The definition of the clear-cell type carcinoma in this classification is different from that in classification A. Chromosome analysis was carried out as described by de Jong *et al.* (1988).

RESULTS

Histological and cytological data could be obtained for 51 RCC tumours. These data, together with data on LOH at chromosome 3 are listed in Table I. LOH data for the first 34 tumours have been described more extensively (van der Hout *et al.*, 1991). Table I shows that LOH at at least one 3p locus was found in 32 cases (43%). When we consider the histological classification, LOH at at least one 3p locus was found in 17 out of 34 solid tumours, in 2 out of 5 tubular tumours, and in 3 out of 7 mixed solid/tubular tumours. No allelic losses were observed in the single papillary and 4 papillary/tubular tumours. Considering cytological classification A, LOH at 3p was found in 14 out of 24 clear-cell tumours, in 7 out of 12 mixed clear-cell/granular-cell tumours, and in the only mixed clear-cell/spindle-cell tumour. No losses were found in the 8 granular tumours and the 6 oncocytomas. Applying cytological classification B, LOH for at least one 3p probe was found in 22

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TABLE I – HISTOLOGICAL AND CYTOLOGICAL TYPING AND LOH AT 3p IN RENAL-CELL TUMOURS

Tumor number[1]	Histology	Cytology A[2]	Cytology B[3]	LOH at 3p
1	Solid	Clear	Clear	+[4]
3	Solid	Clear	Clear	+
4	Solid	Clear/granular	Clear	+
5	Solid	Granular	Clear	–
6	Solid	Clear	Clear	+
7	Solid	Granular	Clear	–
8	Papillary/tubular	Clear	Chromophilic	–
22	Solid	Clear	Clear	+
23	Tubular	Granular	Chromophilic	–
24	Papillary	Granular	Chromophilic	–
25	Solid	Oncocytic	Oncocytic	–
26	Solid	Clear	Clear	+
27	Solid	Clear	Clear	–
28	Solid	Oncocytic	Oncocytic	–
31	Solid	Clear/granular	Clear	–
33	Solid	Clear	Clear	+
34	Solid	Clear	Celar	+
36	Tubular	Clear/granular	Clear	+
37	Tubular	Granular	Clear	–
38	Solid	Clear	Clear	+
40	Tubular	Clear	Clear	–
41	Solid/tubular	Clear/granular	Clear	–
42	Solid/tubular	Clear	Clear	+
43	Solid/tubular	Clear/granular	Clear	+
46	Solid	Clear	Clear	+
47	Tubular	Clear/granular	Clear	+
48	Solid	Clear/granular	Clear	+
49	Solid	Clear	Clear	+
50	Solid	Clear/granular	Clear	–
51	Solid	Oncocytic	Oncocytic	–
52	Solid	Clear/granular	Clear	–
53	Solid	Clear/spindle	Clear	+
55	Solid/tubular	Clear	Clear	–
56	Solid	Clear	Clear	–
57	Solid	Oncocytic	Oncocytic	–
58	Solid	Oncocytic	Oncocytic	–
59	Solid	Oncocytic	Oncocytic	–
60	Papillary/tubular	Granular	Chromophilic	–
61	Papillary/tubular	Granular	Chromophilic	–
62	Papillary/tubular	Granular	Chromophilic	–
63	Solid	Clear	Chromophobic	–
64	Solid/tubular	Clear	Chromophobic	–
65	Solid	Clear	Chromophobic	–
67	Solid	Clear	Clear	+
68	Solid	Clear	Clear	+
70	Solid/tubular	Clear/granular	Clear	+
71	Solid/tubular	Clear	Clear	–
72	Solid	Clear/granular	Clear	+
73	Solid	Clear	Clear	+
74	Solid	Clear	Clear	–
75	Solid	Clear/granular	Clear	–

[1]Tumour numbers refer to those used in van der Hout *et al.* (1991).–[2]Based on WHO classification (Mostofi, 1981).–[3]Classification proposed by Thoenes *et al.* (1986).–[4]+ loss of heterozygosity at at least one locus on 3p; – retention of heterozygosity at all informative loci on 3p.

out of 36 clear-cell tumours, whereas none of the 15 non-clear-cell tumours (chromophilic, chromophobic, and oncocytoma) showed LOH.

Of 36 tumours designated as clear-cell according to classification B, 20 were informative for at least one 3q probe (Table I). Only 2 showed LOH at 3q (cases 53 and 68). Both of these tumours also showed allelic loss at all informative loci at 3p. The only other category showing LOH at 3q was the group of chromophobic cell tumours. Here, 2 out of 2 informative cases (63 and 65) showed LOH at the 3q locus D3S5. Densitometric analysis gave no indication of allelic losses at the more centromeric loci D3S1 and MOX1 in these 2 tumours, or at any of the 3 3q loci in the third chromophobic tumour.

For 30 of the tumours listed in Table I, both molecular and microscopic cytogenetic analyses have been carried out. In 11 cases there were 2 microscopically normal copies of chromosome 3 and accordingly no LOH at 3p was found. Table II shows LOH and cytogenetic data concerning chromosome 3 for the remaining 19 cases. Full karyotypes will be published elsewhere. In 7 cases cytogenetic analysis shows deletions or unbalanced translocations resulting in the loss of (part of) 3p in correspondence with the pattern of allelic losses (cases 1, 4, 6, 36, 42, 46 and 47). In the remaining 12 cases there is an apparent discrepancy between the results of microscopic and molecular analysis. In 6 of these cases there are 2 microscopically normal copies of chromosome 3 whereas molecular analysis shows LOH at 3p (cases 3, 22, 33, 34, 38 and 53). In the other 6 cases microscopic cytogenetic analysis shows abnormalities of 3p that are not reflected, or only partly reflected, as allelic losses by molecular analysis (cases 7, 40, 43, 48, 49 and

TABLE II – MOLECULAR AND CYTOGENETIC DELETION ANALYSIS OF CHROMOSOME 3 IN RCC

Tumor number	Loss of heterozygosity					Chromosome 3 homologues
	3p25	3p24	3p21	3p14	3q	
1			+[1]	+		3, der(3)t(1;3)(q21;p13)
3	+	+	+		○	3, 3
4		+			○	3, del(3)(p11)
6	+		+		○	3, der(3)t(3;5)(p12;q15)
7	○	○				3, 3, 3, der(3)t(3;5)(p13;q15)×2
22	+	+			○	3, 3
33			+		○	3, 3
34	+	+	+	+		3, 3
36	+	+	+			3, del(3)(p21)
38			+			3, 3
40	○		○		○	3, del(3)(p14) and 3, 3
42		+	+	+		3, 3, 3, der(2)t(2;3)(q33;q11)×4
43		+	+	○	○	3, 3, der(3)t(3;5)(p11;q21)
46	+	+	+			3, del(3)(p13 or 14)
47	+	+	+			3, add(3)(p12)
48	○	○	+			3, 3, der(10)t(3;10)(q11;q11)×2
49	○	○	+		○	3, der(3)t(3;5)(p21;q31)
52	○	○				3, der(6)t(3;6)(q21;q13)
53		+	+		+	3, 3

[1]+, Loss of heterozygosity; ○, retention of heterozygosity; no entry: homozygous in normal kidney tissue.

52). Chromosome 3 homologues from these tumours are shown in Figure 1.

DISCUSSION

We compared cytogenetic and molecular deletion analyses in 30 RCC tumours. In 12 cases results of the 2 analyses do not seem to be in agreement. These discrepancies can be readily explained in most cases.

In tumour 53, which shows microscopically normal copies of chromosome 3, but LOH at 3p as well as 3q, one copy of chromosome 3 has presumably been lost and the remaining copy duplicated. In tumours with microscopically normal copies of chromosome 3 and LOH at 3p with retention of heterozygosity at 3q (tumours 3, 22 and 33) the mechanism underlying allelic loss may be mitotic recombination. In tumours 34 and 38 that also have microscopically normal copies of chromosome 3 and LOH at 3p, but for which all 3q probes are uninformative, either chromosome loss followed by duplication of the remaining homologue or mitotic recombination may be applicable.

Cytogenetic results show that there is loss from one chromosome 3 homologue of the short-arm region including bands 3p24 and 3p25 in tumours 7, 48, 49 and 52, whereas molecular data show the presence of 2 different alleles at loci in these bands. Probably the 3p24-25 region has been translocated to some other chromosome, but escaped detection. *In situ* hybridization with a chromosome-3-specific DNA library might validate this suggestion.

In tumour 40, loss of the part distal to 3p14 has been found by microscopic analysis, but only in a subset of tumour cells. No LOH was found at 3p, perhaps because cells without the deletion predominated in the tumour sample from which the DNA was isolated. Molecular analysis of tumour 43 shows retention of heterozygosity at 3q and 3p14 and loss at more distal 3p loci. Cytogenetic results show an unbalanced translocation with the breakpoint most probably in 3p11. However, the quality of the karyotype does not allow a breakpoint in 3p14 to be definitely ruled out. Thus, our comparison of cytogenetic and molecular genetic analysis of 3p in renal-cell tumours indicates that, in the molecular analysis, presumably only rearrangements occurring in a small proportion of tumour cells may be missed. Reduction to homozygosity by mechanisms such as mitotic recombination or chromosome loss followed by duplication of the remaining copy—mechanisms that appear to be quite common in renal-cell tumours—will, however, remain unnoticed upon microscopic analysis.

LOH at locus D3S5 (3q25-28) was found in 2 out of 3 chromophobic tumours, whereas at 2 more proximal 3q loci (D3S1 and MOX1) heterozygosity was retained. In clear-cell tumours, allelic loss at 3q is much more rare. We observed this in 2 out of 20 informative cases. In both cases loss of 3q alleles was probably caused by loss of a whole chromosome 3 homologue because 3p alleles had also been lost. More chromophobic-cell tumours need to be studied to verify whether the deletion of a part of 3q is characteristic of this group of tumours.

The presence or absence of allelic losses at 3p might be related to the reported histopathological heterogeneity in renal-cell tumours. One classification of renal tumours differentiates only between papillary and non-papillary tumours (Kovacs, 1990). LOH has only been found in non-papillary tumours (Kovacs *et al.*, 1989). In our material it was difficult to make a distinction between papillary and non-papillary tumours. Although part of some tumours had a tubulo-papillary character, the occurrence of more than 75% of papillary structures in a tumour—which was the criterion of Kovacs *et al.* (1989) for definition of papillary tumours—was very rare. Only one of our cases met this condition. In most studies renal tumours are classified according to a somewhat confusing mixture of histological and cytological criteria (Ogawa *et al.*, 1991; Presti *et al.*, 1991; Anglard *et al.*, 1991, 1992; Yamakawa *et al.*, 1991). In these studies most cases with LOH on 3p belonged to the category designated clear-cell tumours. In each study, however, there were some cases with 3p deletions in other categories. We tried to apply the histological and cytological criteria, as used in the above-mentioned studies, separately in 2 different classifications—a histological one and a cytological one (A in Table I). Cases with LOH appeared to occur in 3 different histological groups. Using the cytological classification (A), LOH was found both in clear-cell tumours (58% of cases of this group) and in tumours with a mixed cytology of clear cells and granular or spindle cells (62% of cases of this group). A second cytological classification which we applied was that proposed by Thoenes *et al.* (1986) (B in Table I). This classification is based on the different renal-tubule cell types from which the tumours arise. When we apply this classification to our tumour material, the clear-cell cate-

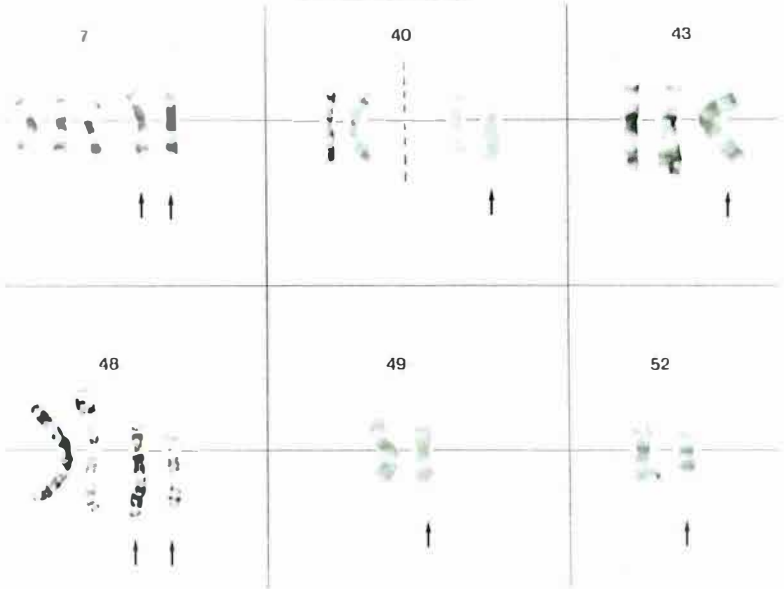

FIGURE 1 – Chromosome 3 homologues from 6 cases showing cytogenetic abnormalities of chromosome 3 which are not or only partly reflected as allelic losses. In case 40, 2 different clones of tumour cells were found. Chromosome 3 homologues from both clones are shown. Arrows point to abnormal chromosomes. Rearrangements are described in Table II.

gory covers all cases with LOH on 3p. The frequency of LOH in this group is 61%. It is unlikely that the absence of LOH in non-clear-cell tumours would be due to random variation. Although the number of non-clear-cell tumours studied is lower than the number of clear-cell tumours, the mean number of informative 3p probes per case in the non-clear-cell tumours was even higher than in the clear-cell tumours (3.6 *vs.* 3.3).

The most noticeable difference between both cytological classifications that we applied (A and B in Table I) is that all mixed clear-cell/granular-cell tumours in classification A appear as clear-cell tumours in classification B. This is because tumour cells which contain numerous mitochondria, and thus have a granular appearance causing their inclusion as granular-cell tumours in classification A, also may have clear-cell properties, causing their inclusion as clear-cell tumours in classification B.

The relation between the histological classification of Kovacs and the cytological classification of Thoenes is that all tumours of the chromophilic category in the cytological classification have a tubulo-papillary growth pattern (Thoenes *et al.*, 1986, tumours 8, 23, 24, 60, 61 and 62 in Table I). Neither the cytological category of chromophilic tumours nor the histological category of papillary tumours show LOH at 3p. Thus, it is possible that all tumours designated papillary in other studies have a chromophilic cell type (*e.g.*, tumour 24 in Table I).

When more probes closer to the presumed locus of a tumour-suppressor gene are applied, LOH at 3p may possibly be found in all renal tumours. In that case, the deletions, as found in clear-cell tumours up to now, are the larger deletions. Alternatively, this may only apply to clear-cell tumours, while non-clear-cell tumours may have a different genetic background. Nevertheless, a common (yet unknown) first step in all types of renal-cell tumour, followed in clear-cell tumours by LOH of 3p, cannot be excluded. Morita *et al.* (1991) also suggested the existence of an alternative pathway for the development of RCC, without the involvement of genes on 3p. They based their conclusion on the finding of LOH on other chromosomes in tumours that retained heterozygosity at all informative loci at 3p.

Whatever the mechanisms involved, a search for smaller 3p deletions that would pinpoint the location of a chromosome 3 tumour-suppressor gene should primarily be aimed at those clear-cell tumours in which no LOH has been detected with the probes used so far.

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4.3 Cytogenetic analysis of epithelial renal cell tumours: relationship with a new histopathological classification

(submitted for publication)

Cytogenetic analysis of epithelial renal cell tumours: relationship with a new histopathological classification

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Summary

Renal cell carcinomas (RCC) are clinically, histologically and cytogenetically very heterogeneous. The actual histological WHO classification shows no clear correlation between histologic subtypes and specific chromosomal abnormalities. In 1986, a new classification was proposed by Thoenes and Störkel based on the cell type from which the tumour arises. They distinguish 5 cell types: clear cell-, chromophilic, chromophobic-, ductus Bellini- and the oncocytic type. Results of 105 primary tumours show that in this new classification there is a correlation between different subtypes of renal cell tumours and specific chromosomal abnormalities at a microscopic and/or molecular level. The clear cell compact type has structural aberrations of chromosomes 1, 3, 4, 5q, 6, 10q, 11q, and 12q together with polysomy of chromosomes X, 4, 5, 7, 10, 12, 15, 16, 19, 20, 21, and 22 and monosomy of chromosomes 3, 8, 9, 13, 14, and loss of Y. The main characteristics of the chromophilic tubulo-papillary type are trisomies 7 and 17, and loss of the Y-chromosome. Chromophobic carcinoma seems to be correlated with, amongst others, polysomy 7, trisomies 12, 16, 18, 19, structural abnormalities of 11q, and telomeric associations. Oncocytomas do not reveal any specific chromosomal anomaly, except for trisomy 7. Loss of heterozygosity on 3p is only found in the clear cell compact type. Some specific chromosomal abnormalities correlate with a particular grade of the tumour. These correlations support the hypothesis that specific chromosomal abnormalities seem to play a role in the histogenesis and oncogenesis of RCC. They may be important for the diagnosis of the tumour and the prognosis of the patient.

Introduction

Renal cell carcinoma (RCC) is the most common malignant tumour arising in the kidney and accounts for 2% of all new cancers diagnosed. The incidence of RCC

increases with each decade of life and has a peak incidence in the sixth decade of life (Medeiros and Weiss,1990). Survival correlates best with the stage of the tumour at nephrectomy.

Based on macroscopic, histological, and ultrastructural features the current WHO classification subdivides renal tumours into adenomas, carcinomas, and other tumours (Mostofi,WHO,1981). At a cytological level clear cells, granular cells, spindle cells, oncocytic cells, and tumours with mixed cell types are distuinguished (Mostofi,1981). This classification provides little insight into the oncogenesis, progression, and clinical behavior of these tumours (van der Hout et al.,1993; Thoenes et al.,1986).

Based on new electron microscopic and histochemical observations, a different approach in the classification of renal cell tumours was suggested by Thoenes et al. (1986;1990a). This classification assumes that the various subtypes of renal cell carcinoma derive from different cells of origin in different parts of the nephron (Thoenes et al.,1986; Thoenes et al.,1990b; Störkel and Jacobi,1989). Five basic cell types are distuinguished: (1) clear cell and (2) chromophilic cell type, both derived from the proximal tubule; (3) chromophobic and (4) oncocytic cell type, derived from the intercalated cells type B and A from the cortical collecting duct, respectively; and (5) Duct Bellini cell type derived from the principal cells of the medullary collecting duct. Three growth patterns which can be deduced from the tubule, can be distinguished: (1) compact (subtype: acinar); (2) tubulopapillary, and (3) cystic. There is a relationship between growth pattern and cell types, although not exclusive: clear and chromophobic cell type are predominantly related to compact growth, chromophilic to tubulopapillary growth, oncocytic (true 'renal oncocytoma') is related to acinar, and Bellini duct is related both to compact and (tubulo)-papillary growth.

RCCs are graded with respect to nuclear atypia including the size of nucleoli, supplemented by cytoplasmic features, e.g. diminution of basic features, augmentation of eosinophilia/granularity (=number of mitochondria), and spindle/pleomorphic cell form.

Numerous reports have appeared in the literature on the cytogenetic analysis of renal cell tumours (a.o. De Jong et al.,1988; Kovacs et al.,1989; Walter et

al.,1989; Presti et al., 1992; Meloni et al.,1992a). The most frequent finding is loss of the short arm of chromosome 3. Other frequent observations are gain of chromosomes 5, 7, 12, 16, and part of the long arm of chromosome 5, loss of chromosomes 8, 13, 14, Y, as well as structural abnormalities of the chromosomes 1, 3, and 5. Certain specific chromosomal abnormalities may correlate with different histological subtypes of renal tumours (Dal Cin et al.,1989; Presti et al.,1991; Kovacs et al.,1991). However, cogent correlations with cytogenetic findings can seldom be made, possibly because the WHO classification does not adequately distuinguish between the various histological subtypes of RCC.

In the present study of 105 primary renal cell tumours we try to correlate the histopathology of the tumour described according to the classification of Thoenes and Störkel (1986) with the cytogenetic results. The finding of specific chromosomal abnormalities in different types of renal cell tumours might contribute to a better understanding of the biology of this group of tumours. Also it might have a diagnostic and prognostic value, as Störkel et al. (1989,1990) already indicated.

Materials and methods

Histopathology

Fifty-two specimens of primary RCC were obtained from the department of Urology, University Hospital Groningen, 49 specimens from the department of Pathology, Johannes Gutenberg University, Mainz (Germany), 1 case of oncocytoma (kindly provided by dr. G. Brutel de la Rivière) from the department of Pathology, Martini Hospital, Groningen and three cases of chromophobic carcinoma (kindly provided by dr. W. Henn) from the department of Human Genetics from the University of Homburg, Homburg (Germany).

Diagnostic specimens were formalin-fixed and paraffin-embedded. Sections of 5µm-thickness were stained with hematoxylin and eosin. Additional stainings for the chromophobic type were performed if necessary. Except for the German cases, the specimens were independently reviewed by two pathologists (S.S. and J.W.O.),

without prior knowledge of cytogenetic results. Each tumour was characterized for cell type, histological pattern, and pathological grading using the classification according to Thoenes and Störkel (1986).

Tissue culture and chromosome analysis

Tumour specimens were obtained from radical nephrectomy at surgery. About 1 g of tissue was disaggregated by mincing with scalpels and incubated in collagenase II, 250 units/ml (Worthington Diagnostic Systems Inc., Freehold, NY) and DNAse 0.002% (Sigma Chemical Company, St. Louis, Mo) for 1 to 2 h. The disaggregated cells were washed twice with medium (RPMI 1640, supplemented with 13% fetal calf serum, glutamine, 1% sodiumpyruvate and antibiotics), seeded in T75 tissue culture flasks (Costar, Cambridge, MA), and cultured in a 5% carbon dioxide incubator at 37 ^{o}C in a humidified atmosphere. Chromosome preparations of tumour cells were made after short-term (3-14 days) culture, using standard cytogenetic techniques. The chromosomes were GTG banded. DNA-ploidy was determined by means of flow cytometry in all but the German cases.

Analysis of loss of heterozygosity (LOH) on 3p

DNA analysis has already been described by van der Hout et al. (1993). Because of limited availability of some, not all tumours have been included in this analysis.

Results

We karyotyped 105 primary renal tumours, that were classified as follows: 79 clear cell, 11 chromophilic, 10 chromophobic tumours, and 5 oncocytomas. In Fig.1 a selection* is given of the most significant numerical and structural chromosomal aberrations in different morphological subtypes of RCC as proposed by Thoenes et al. (1986). Only those clonal chromosomal aberrations have been

Fig.1 Most significant (present in ≥10% of the tumours) chromosomal abnormalities in different morphological subtypes of RCC, according to the histopathological classification as proposed by Thoenes et al. (1986). Indicated between parentheses in the columns are the number of tumours with these specific aberrations. Molecular data from van der Hout et al. (1993).

included that were present in at least 10% of the tumours within each subtype per grade.

Table 1. *Frequency of occurrence of clonal chromosomal abnormalities in the clear cell type tumours per grade. (n=79; ♂=45)*

GRADE	N	NUMERICAL GAIN	NUMERICAL LOSS	STRUCTURAL
1	8 (3)	7 (1)	3 (1)	1 (2) 3p (4) 5q (2)
2	44 (24)	5 (5) 7 (20) 12 (5)	3 (6) 8 (8) 9 (7) 13 (7) 14 (10) Y (7)	1 (8) 3p (21) 5q (13) 6 (5)
3	27 (18)	4 (3) 5 (4) 7 (15) 10 (5) 12 (4) 15 (3) 16 (3) 19 (3) 20 (3) 21 (3) 22 (4) X (3)	8 (4) 13 (4) 14 (6) Y (8)	1 (6) 2 (3) 3p (9) q (6) 4 (4) 5q (3) 6q (5) 10q (3) 11q (4) 12q (3)

Between parentheses in the column with the number of cases are indicated the number of male patients. Between parentheses in the other colums the number of tumours with these specific aberrations is given.

Our principal findings can be summarized as follows: the clear cell type (Figure 1 and Table 1) is characterized by structural abnormalities of chromosomes 3p (43% of the tumours), 5q (23%), 1 (20%), 6 (13%), 3q (8%), 4 and 11q (5% each), 10q and 12q (4% each), as well as by polysomy 7 (46%), 5 and 7 (11% each), 10 (6%), 22 (5%) and 12, 15, 16, 19, 20, 21 (each 4%), and monosomy 14 (20%), 8 (15%), 9 and 13 (14% each), 3 (9%), and loss of the Y-chromosome in males (33%). Fifteen out of 79 (19%) clear cell tumours have translocations between the short arm of chromosome 3 and the long arm of chromosome 5. Breakpoints are situated in regions 3p11-21 and 5q13-5q31. All clear cell tumours were of the compact growth type. Twenty-two out of 36 clear cell tumours (61%) showed LOH (loss of heterozygosity) on 3p.

Table 2. *Frequency of occurrence of clonal chromosomal abnormalities in the chromophilic tumours per grade. (n=11 ; ♂=10)*

GRADE	N	NUMERICAL GAIN	NUMERICAL LOSS	STRUCTURAL
1	2 (2)	7 (2) 16 (2) 17 (2)	Y (2)	3q (1)
2	7 (6)	7 (4) 17 (2)	Y (5)	1 (3) 3q (1)
3	2 (2)	7 (2)	Y (1)	

Between parentheses in the column with the number of cases are indicated the number of male patients. Between parentheses in the other colums the number of tumours with these specific aberrations is given.

Some chromosomal abnormalities are only found in particular grades of the tumour as shown in Table 1. DNA ploidy varied from peridiploid to highly aneuploid (0.88 - 3.16) in keeping with the chromosome numbers observed by karyotyping (40-111 chromosomes), except for four cases where we presumably did not

Table 3. *Frequency of occurrence of clonal chromosomal abnormalities in the chromophobic tumours per grade. (n=10 ; ♂=7)*

GRADE	N	NUMERICAL GAIN	NUMERICAL LOSS	STRUCTURAL
1	1 (1)	3 (1) 7 (1) 12 (1) 16 (1) 18 (1) 19 (1) 20 (1)		
2	7 (6)	3 (2) 5 (2) 7 (3) 8 (2) 12 (3) 16 (3) 18 (3) 19 (3) 20 (3)	Y (2)	3p (1) 11q (1)
3	2 (0)			3q (1) 11q (1)

Between parentheses in the column with the number of cases are indicated the number of male patients. Between parentheses in the other colums the number of tumours with these specific aberrations is given.

aryotype tumour cells but fibroblasts or normal kidney parenchyma cells.

The chromophilic cell type (Figure 1 and Table 2), on the other hand, is associated with much less chromosomal aberrations. In a total of eleven cases only trisomies 7 (40%), 16 (20%), 17 (40%), and also loss of the Y-chromosome (80% of the males) are often found as well as structural aberrations of 1 (30%) and 3q (20%). All but one chromophilic tumours had the tubulo-papillary growth type; the exception was a compact growing one. DNA ploidy was in keeping with the chromosome number and in all but one case diploid. None of six specimen subjected to DNA analysis did show LOH on 3p.

In ten cases of chromophobic carcinoma with a compact growth pattern (Fig. 1 and Table 3), the only frequent finding seemed polysomy 7 and structural aberrations of 11q (two cases) as well as trisomy 12, 16, 18, 19, and 20. For only 3 cases an LOH analysis on 3p was carried out. No LOH on 3p was found.

Table 4. *Frequency of occurrence of clonal chromosomal abnormalities in the oncocytic tumours. (n=5 ; ♂=4)*

N	NUMERICAL		STRUCTURAL
	GAIN	LOSS	
5 (4)	7 (2)	Y (1)	1p (1) 5q (1) 11q (1) 19q (1)

Between parentheses in the column with the number of cases are indicated the number of male patients. Between parentheses in the other colums the number of tumours with these specific aberrations is given.

Finally, the five cases of oncocytoma that were karyotyped, did not have any specific chromosomal anomaly, except for trisomy 7 (Fig. 1 and Table 4). In oncocytoma LOH analysis on 3p did not reveal any loss. DNA ploidy was diploid in accordance with the chromosome number.

**A complete description of the karyotypes of all RCCs is available from the first author.*

Discussion

We analyzed the cytogenetic changes in 105 cases of RCC in relation to the histopathological subtypes as classified according to Thoenes et al. (1986). Our results suggest that some chromosomal abnormalities are (more or less) characteristic for a specific subtype or tumour grade (Tables 1-4), whereas other changes are non-specific, occurring in all subtypes (Fig. 1).

Loss of chromosome 3 or structural abnormalities of the short arm of chromosome 3 resulting in a deletion of region 3p11-21 occurred in 52% of tumours of the clear cell type. DNA analysis, carried out on 36 tumours resulted in a proportion of 61% for LOH on 3p. High percentages of LOH on 3p have also been described by other groups (reviewed in van der Hout et al.,1993). In microscopic cytogenetic studies, it cannot be excluded that the percentage of cases with 3p deletions is an underestimation, because morphologically normal copies of any chromosome pair may represent copies of a single homologue having duplicated itself after loss of the other homologue. Therefore, LOH and microscopic deletions of 3p will not always coincide. Another reason for discrepancy between microscopic and molecular analyses will be discussed later on.

Gain of chromosome 5 or structural anomalies of the long arm of chromosome 5, resulting in a partial trisomy for the long arm of chromosome 5, also seems to be characteristic for clear cell carcinoma as already discussed at length by Kovacs et al. (1988) and Kovacs and Frisch (1989). Whereas in Kovacs' studies the breakpoint most often was in 5q22, in our cases it was in 5q15. Structural aberrations of 5q, 6q, and 10q were often found in grade 2 and 3 of clear cell tumours. This is in accordance with the work by Morita et al. (1991), who also found that LOH on 5q, 6q, and 10q is more frequent in grade 2 and 3 clear cell tumours than in grade 1 tumours (Table 1). Anglard et al. (1991) reported a relationship between LOH on 13q and progression of the tumour, which seems to be supported by our finding of loss of chromosome 13 (Table 1). Loss of chromosomes 8, 9, and 14 (Kovacs and Frisch,1989; Maloney et al.,1991), especially the combination of -8,-14 (our results), might reflect tumour progression in the clear cell carcinomas, because it occurred only in grade 2 and 3 tumours.

No specific changes were found in tumours of the chromophilic cell type, although trisomy 17 was [often] found, usually in combination with trisomy 7 (Table 2). In our results trisomy 17 seems not to be correlated with the chromophilic cell type per se, but with tubulopapillary growth (which is often present in chromophilic tumours). This is illustrated by the fact that we find trisomy 17 in clear cell type tumours with a tubulopapillary growth pattern (results to be published elsewhere). Kovacs et al. (1991) also suggested a specific involvement of trisomy 17 in papillary RCCs. No LOH on 3p was detected in chromophilic tumours.

Kovacs et al. (1992b), reported LOH on 3p in 5 out of 9 cases of chromophobic tumours. We analyzed a chromophobic tumour which revealed four copies of chromosome 3 and a der(X)t(X;3)(p22;p13). However, the region 3p13→3pter was retained in this case. On microscopic level no LOH on 3p was found. Unfortunately, DNA analysis was not possible. LOH on 3p may occur in at least two types of RCC in contrast to our and other groups previous reports (a.o. Ogawa et al.,1991; Anglard et al.,1991; Presti et al,1991; Yamakawa et al.,1991; van der Hout et al.,1993).

Only few cases of chromophobic carcinomas with karyotypic anomalies have been published (Kovacs et al.,1988; Kovacs and Kovacs,1992a; Crotty et al.,1992). They revealed low chromosome number, telomeric association, and pulverisation of chromosomes. From these publications the loss of 1, 2, 6, 10, 13, 17, and 21 as well as telomeric associations seem to be characteristic for chromophobic carcinoma. Loss of Y was also noted. One of our cases fits the description, but has additional loss of 11, 14, and 18 (Table 3). This tumour had also a t(11;19)(q12;p12) and a del(3)(q13). Similar numerical aberrations have been reported in a cytogenetic characterization of three cases of collecting duct carcinoma with monosomies of chromosomes 1, 6, 14, 15, and 22, resulting in a low chromosome number of the tumour (Füzesi et al.,1992). In view of the aberrations this tumour would fit in the chromophobic type or it may be that loss of chromosomes 1 and 6 is a joint characteristic of chromophobic and other collecting duct tumours (Ductus Bellini carcinoma and oncocytoma). Furthermore, telomeric associations were observed in two chromophobic tumours. It is remarkable that chromophilic as well as chromophobic tumours show breakpoints in

the long arm of chromosome 3 (Tables 2 and 3: 3q11, 3q13.3 and 3q13, respectively). Van der Hout et al. (1993) reported LOH on 3q in two out of two informative chromophobic tumours. Whether these 3q rearrangements have any specific significance remains to be clarified.

Except for the combination of -Y, -1 in seven cases (Miles et al,1988; Psihramis et al.,1988; Meloni et al.,1992b; Crotty et al.,1992; Dobin et al.,1992), no common chromosomal anomalities have been described in oncocytomas. None of the 5 oncocytomas in our series (Table 4) had a monosomy 1, and loss of Y occurred only once. We observed a t(5;11)(q35;q13) which was also present in a case of oncocytoma published by Presti et al. (1991). Structural aberrations of 11q were also observed in two of our chromophobic tumours. They had different breakpoints which also differed from the oncocytoma breakpoint. Nevertheless, it might be that 11q aberrations are common to both chromophobic carcinomas and oncocytomas and thus related to their 'common' origin, although from different cell types of the cortical connecting duct.

Unfortunately, we were not able to karyotype a Ductus Bellini carcinoma.

As the above suggestions have been based upon relatively small numbers of observations, they still await further evaluation. From our results it is clear that, in general, only a limited number of metaphases turn out to have the more or less consistent abnormalities for the type of tumour represented by a given specimen. This implies that a microscopic analysis per cell is the only way to detect these abnormalities. In an analysis of DNA extracted from the whole sample aberrations would be masked by the large majority of cells in which they are absent. This is another explanation for discrepancies between microscopic analysis and DNA analysis apart from the one given earlier.

In all subtypes of RCC trisomy 7 and loss of Y could be observed (Fig. 1). Trisomy 7 might be related to amplification and overexpression of the epidermal growth factor receptor gene (EGFR) located on chromosome 7. Ishikawa et al. (1990) showed such an overexpression in 60% of RCC cases without a correlation between this phenomenon and tumour grade. The combination of trisomy 7 and 10 is seen in four cases of metastatic renal cell carcinoma and might be associated with tumour progression (unpublished results). Elfving et al. (1992) observed that the frequency of trisomy 7 did not depend on the length of the culture

period, nor was it influenced by the presence or absence of epidermal growth factor in non-neoplastic kidney tissue from 4 patients of whom 3 had a RCC. It remains, however, uncertain whether these abnormalities are specific for RCC. Trisomy 7 (and 10) and loss of Y have been demonstrated to be present to be present in normal cells of tumour-adjacent kidney parenchyma rather than in the tumour itself (Emanuel et al.,1992). Dal Cin et al.,1992) showed that trisomy 7 and trisomy 10 can be found in subpopulations of tumour-infiltrating lymphocytes. Recently, occurence of trisomies 5, 8, and 18 has also been reported for non-neoplastic kidney tissue (Casalone et al.,1992). Trisomy 7 and loss of Y in our specimens may, therefore, have limited or no significance with respect to RCC development and progression. Alternatively it might be considered as a preneoplastic change in renal parenchymal cells.

Specific differences have been observed in chromosomal pattern between renal cell adenomas (relatively benign) and renal cell carcinomas. The combination of tri- or tetrasomy 7 and trisomy 17 as the only autosomal abnormalities might be specific for benign papillary renal cell adenomas or renal cortical adenomas (Dal Cin et al.,1989; Kovacs et al.,1991). Kovacs (1991) reported that malignant papillary renal cell carcinomas are characterized by additional trisomies of the chromosomes 12, or 16, or 20, alone or in combination. In our series we have one chromophilic grade 1 tumour showing the -Y,+7,+7,+17 combination as well as trisomies of chromosomes 16 and 20. Hence, this tumour might demonstrate progression from a benign to a malignant tumour. However, the same trisomies of chromosomes 12, 16 and 20 are also found in clear cell grade 3 tumours as well as in the (less malignant) chromophobic type (Tables 1-3).

In conclusion, our results demonstrate that certain chromosomal aberrations or combinations of aberrations correlate with the histological subtyping of RCC according to Thoenes et al. (1986). Moreover, they indicate that relations exist between the origin and progression of this heterogeneous group of tumours and cytogenetic aberrations. To establish further relationships more tumours need to be analyzed. A possible prognostic value of these specific cytogenetic aberrations or patterns of aberrations has to be determined from a detailed clinical analysis.

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SUMMARY

This thesis describes the search for genes whose functional elimination would be responsible for the development of renal cell carcinoma (RCC). In Chapter 1 an overview is given of the different forms of RCC. RCC can occur sporadically or in a hereditary form, either as pure familial RCC or as one of the tumours which are characteristic for the von Hippel-Lindau syndrome (VHL). In all three forms cytogenetic studies indicate a specific loss of a part of the short arm of chromosome 3 (3p). In a family with pure familial RCC a constitutional translocation (3;8)(p14.2;q24) seems to predispose to RCC. Linkage analysis in families affected with VHL localized the gene for this syndrome in 3p25-p26. Epidemiological studies have indicated that a model, according to which a tumour develops as a consequence of the functional loss of both alleles of a tumour suppressor gene, may be applicable to RCC. Such a tumour suppressor gene should be located on 3p, the chromosome region which appears to play a role in the predisposition to hereditary RCC and which most often is found deleted in the tumours.

Chapter 2 describes the study of loss of heterozygosity on 3p in sporadic RCC to define the region in which to look for a tumour suppressor gene. We found deletions in 50% of the tumours and defined the region 3p21-p24 as the region common to all deletions. Since this chromosome segment does not include the location of the VHL gene, nor the 3p breakpoint of the aforementioned translocation predisposing to hereditary RCC it is concluded that different genes must be involved in the development of sporadic RCC, pure familial RCC, and VHL. For some genes located in 3p21-p24 a tumour suppressor function has already been considered, because of decreased levels or total absence of the mRNAs of these genes in certain tumour types. We found that in RCC tumours and tumour cell lines the mRNA levels for two of these genes, D8 and APEH, did not deviate from what we found in normal kidney cells. Therefore, we do not consider these genes to be likely candidate tumour suppressor genes in RCC.

Chapter 3 focuses on the chromosome region 3p14. We aimed at cloning the region around the 3p breakpoint in the previously mentioned t(3;8) associated

with hereditary RCC, to see whether this translocation has affected some gene which therefore could be responsible for hereditary RCC. Three widely used 3p probes were localized with respect to the breakpoint by in situ hybridization. A probe from the locus D3S3 failed to hybridize to either of the two translocation chromosomes. We considered this as an indication that D3S3 might be close to the breakpoint. Therefore, we constructed a long-range restriction map around D3S3. This became only possible after we treated cells from which we wanted to isolate DNA with a demethylating agent because the region around D3S3 appeared to be heavily methylated. However, there was no indication for the presence of the t(3;8) breakpoint within the approx. 1 Mb covered by this map. Probes that were more close to the breakpoint were isolated from a 3p14 specific microdissection library. Unique sequence clones from this library were used for long-range restriction mapping. The t(3;8) breakpoint was defined between two groups of clones, one distal to the breakpoint in 3p14.2 and one proximal in 3p14.1-p14.2. In the 3p14.1-p14.2 region a long-range restriction map covering 4.6 Mb was constructed. However, we found no indications that the breakpoint is located within these 4.6 Mb.

In Chapter 4 the use of somatic cell genetic criteria in the classification of renal cell tumours is discussed. It appeared that in our material loss of heterozygosity on 3p was only found in tumours that histopathologically could be classified as clear cell tumours. This may indicate that only clear cell tumours arise as a consequence of loss of a gene on 3p, or that 3p rearrangements in non-clear cell tumours are more subtle, and therefore not detectable with the set of probes we used. Chromosome studies in tumours belonging to different histopathological subtypes revealed that different subtypes of renal cell tumours are characterized by certain combinations of chromosome abnormalities. Moreover, there seems to be a relation between the occurrence of a certain combination of chromosome abnormalities and tumour progression in specific types of RCC.

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Samenvatting

Op zoek naar genen verantwoordelijk voor het ontstaan van nierceltumoren.

Tumoren van de nier kunnen een verschillende oorsprong hebben. De zogenoemde nierceltumoren, waarover dit proefschrift gaat, ontstaan uit de cellen van het nierepitheel. Het zijn de meest voorkomende tumoren van de nier bij volwassenen. Nierceltumoren komen in onze samenleving voor in een frekwentie van 5 tot 10 per 100.000 personen, meestal als geïsoleerde (sporadische) gevallen. De meeste patiënten zijn op het moment dat de diagnose gesteld wordt ouder dan 50 jaar. Er zijn ook enkele families bekend waarin de aanleg voor nierceltumoren van ouders op kinderen overerft. Daarnaast bestaat nog een erfelijke aandoening, het von Hippel-Lindau syndroom, waarbij de patiënten een sterk verhoogde kans hebben bepaalde tumoren te ontwikkelen, waaronder nierceltumoren.

Kanker ontstaat als gevolg van specifieke veranderingen in het erfelijk materiaal, het DNA. Elke tumorsoort heeft zijn eerste afwijking op een eigen karakteristieke plaats op het DNA. Deze verandering zal in alle cellen van de tumor terug te vinden zijn. Daarnaast ontstaan tijdens de verdere ontwikkeling van de tumoren andere, meer algemene afwijkingen in het DNA. In dit proefschrift wordt beschreven hoe wordt gezocht naar de plaats op het DNA ("het nierceltumorgen") waar de eerste verandering optreedt die van een normale niercel de voorloper van een niertumor maakt.

Relatief grootschalige veranderingen in het erfelijk materiaal kunnen worden opgespoord door de chromosomen van tumorcellen te bekijken. Uit dergelijk cytogenetisch onderzoek was al bekend dat de chromosoomafwijking die het vaakst voorkomt bij nierceltumoren, het verlies van een deel van de korte arm van chromosoom 3 (3p) is. Het verlies van een stuk genetisch materiaal wordt deletie genoemd. Voor verschillende types tumoren is verlies van een specifiek chromosoomgebied gevonden. Een hypothese die al in enkele gevallen bewezen is, voorspelt dat in zo'n chromosoomgebied een gen ligt, dat als functie heeft te voorkomen dat een cel ongecontroleerd gaat delen. Zo'n gen heet tumor suppressor gen. Op 3p wordt dus een niercel-tumor suppressor gen verwacht.

Genen komen voor in twee kopieën, één op een chromosoom dat van moeder afkomstig is, één op een vergelijkbaar chromosoom dat van vader afkomstig is. Voordat een tumor kan ontstaan, moeten in één cel beide kopieën van een tumor suppressor gen uitgeschakeld worden. In het geval van erfelijke tumoren geeft een van de ouders al een defecte kopie van het gen aan het nageslacht door, zodat nog slechts in een willekeurige cel de tweede kopie van het gen defect hoeft te raken. Hoofdstuk 1 van dit proefschrift is de algemene inleiding waarin dit alles staat uitgewerkt.

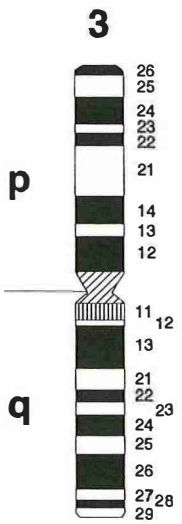

Fig.1.
Chromosoom 3

In de paragrafen 1, 2 en 3 van hoofdstuk 2 wordt beschreven hoe op DNA nivo de grootte van de 3p deleties in sporadische nierceltumoren wordt onderzocht, en wat het kleinste gebied is waarin de deleties elkaar overlappen. Als elk van deze deleties gepaard gaat met het verdwijnen van een tumor suppressor gen dan moet dit gen in het gebied liggen waarin de deleties elkaar overlappen. In de helft van de 58 onderzochte tumoren kon verlies van stukken van 3p aangetoond worden. Het gebied waarin de deleties elkaar overlappen bleek de chromosoombanden 3p21-3p24 te beslaan. (De nummering van de chromosoombanden is aangegeven in Fig.1.) Dit gebied is nog zo groot, dat het wel een paar honderd genen kan bevatten. Het omvat echter niet de gebieden waar het gen voor het von Hippel-Lindau syndroom (VHL) en het gen voor erfelijke nierceltumoren worden verwacht. Koppelingsonderzoek in families met VHL heeft namelijk aangetoond dat het VHL gen in het gebied 3p25-3p26 moet liggen. Onderzoek in een familie met erfelijke nierceltumoren heeft aangetoond, dat alle familieleden die de tumor ontwikkelden, van één van hun ouders een chromosoom 3 hebben geërfd dat gebroken is in band 3p14. Er wordt bijgevolg aangenomen dat door deze breuk een gen wordt uitgeschakeld. Het gen verantwoordelijk voor erfelijke nierceltumoren zou dus in chromosoomband 3p14 liggen. Doordat verschillende localisaties gevonden worden voor respectievelijk de genen voor sporadische nierceltumoren, voor erfelijke nierceltumoren, en voor VHL, moeten er dus op 3p minstens drie verschillende genen liggen die met het ontstaan van nierceltumoren te maken hebben.

Binnen het gebied 3p21-3p24, waar dus een gen verantwoordelijk voor sporaische nierceltumoren moet liggen, zijn 3 genen bekend waarvoor de mogelijkheid s geopperd dat zij een tumor suppressor functie hebben. In paragraaf 2.4 wordt eschreven waarom het niet waarschijnlijk is dat één van deze genen het gezochte en voor sporadische nierceltumoren is.

Hoofdstuk 3 behandelt het chromosoomgebied 3p14, waar als gezegd, mogelijk en gen voor erfelijke nierceltumoren ligt. In het onderzoek is geprobeerd DNA fragmentjes (merkers) in handen te krijgen zo dicht mogelijk bij het breekpunt op 3p in de eerder genoemde familie met erfelijke nierceltumoren. De merker die het dichtst bij het breekpunt ligt, kan dan gebruikt worden als uitgangspunt om genen uit het betreffende gebied te isoleren. De merker D3S3 leek in eerste instantie dicht bij het breekpunt te liggen (paragraaf 3.2). Nadat de omgeving van D3S3 in kaart gebracht was, bleek het breekpunt hier echter niet te liggen (paragraaf 3.3). In paragraaf 3.4 wordt de karakterisering beschreven van merkers die zijn verkregen door de band 3p14 uit enkele chromosomen 3 uit te snijden en daaruit DNA te isoleren. Met deze methode krijgt men de beschikking over een groot aantal merkers uit een betrekkelijk klein gebied. Met behulp van deze merkers is het betreffende gebied gedeeltelijk in kaart gebracht en zijn merkers aan te wijzen die aan weerszijden van het breekpunt dichtbij gelegen zijn. Deze kunnen worden gebruikt als bruggehoofd om een groter DNA fragment in handen te krijgen dat het breekpunt overspant. Op een dergelijk fragment kan dan naar genen gezocht worden.

Nierceltumoren vormen, histopathologisch gezien, een heterogene groep tumoren. In hoofdstuk 4 wordt behandeld in hoeverre genetische kenmerken (3p deleties, specifieke chromosoomafwijkingen) kunnen bijdragen aan een betere classificatie van deze tumoren. Het nauwkeurig vaststellen om welk subtype het gaat kan van belang kan zijn voor de behandeling en voor het voorspellen van het verloop van de ziekte. In paragraaf 4.2 wordt beschreven dat bij de door ons onderzochte tumoren 3p deleties alleen voorkomen in tumoren van het heldercellige subtype. Dit kan betekenen dat de andere subtypes niet ontstaan door het verlies van een gen op 3p, of dat de 3p deleties in deze subtypes veel kleiner zijn en daardoor met de door ons gebruikte merkers nog niet gevonden kunnen worden. In paragraaf 4.3 worden combinaties van chromosoomafwijkingen beschreven

die specifiek bij bepaalde subtypen voorkomen. Ook lijken bepaalde combinaties van afwijkingen alleen voor te komen in tumoren die zich verder ontwikkeld hebben in een meer kwaadaardige richting.

CURRICULUM VITAE

27 april 1959	geboren te Groningen
juni 1977	eindexamen Atheneum B aan het Thorbecke College te Groningen
november 1984	doctoraalexamen biologie aan de Rijksuniversiteit Groningen
januari 1985	aanstelling als onderzoeker bij de vakgroep Medische Genetica (destijds Anthropogenetica) van de Rijksuniversiteit Groningen, op een project: Localisatie van nucleinezuurvolgorden met behulp van in situ hybridisatie, ten laste van de J.K. de Cock Stichting
januari 1987	aanstelling op een project: Microdissectie en microkloning van metafase chromosoomsegmenten; toepassing bij tumorigenese onderzoek aan longcarcinomen en nierceladenocarcinoom, ten laste van de J.K. de Cock Stichting
januari 1989	aanstelling op een project: Identification of a differentiation gene involved in the development of renal cell cancer, ten laste van de Nederlandse Kankerbestrijding
januari 1993	aanstelling ten laste van de Stichting voor Erfelijkheidsvoorlichting te Groningen

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