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Analysis of genomic alterations on urological malignancies by fluorescence in situ hybridization

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Abstract An author (H.M.) had a chance to go abroad to Karolinska Institute, Sweden from 1992 to 94, and learned molecular cytogenetic techniques. The following results had been obtained and published in the literature:

Bladder cancer: Numerical alterations on chromosome 7, 9, 10 and 17 by dual-color fluorescence in situ hybridization (FISH) demonstrated that chromosome 7 trisomy and 9 monosomy were the most frequently occurred not only in the tumor, but also in the surrounding intact bladder mucosa, and that 9 monosomy detected by using negative cytology specimen could predict early recurrence of superficial bladder cancer. Chromosome 17p 13.1 region, on which p53 gene was located, was studied by using cosmid probe, and deletion of these regions had a striking impact on the functional loss of tumor suppressor function in invasive bladder cancer. Immunohistochemical staining of p53 showed clinical significance in predicting patient prognosis in bladder cancer.

Prostate cancer: Studies on chromosomal deletions of 8p22, 23-pter, 10q24-qter, and 16q24 demonstrated that 8p deletion had close association with tumor stage as well as pathological grade. Further investigation on Japanese prostate cancer suggested that putative tumor suppressor genes are located on 8p 21.1-21.2 and 21.3-22. Collaborative studies with Karolinska Institute were published that 16q 24 deletion had significant relation to metastatic ability in prostate cancer, and sporadic prostate cancer arose from mutual influence of these genomic alterations with environmental factors. Further follow-up study proved deletion on 8p22 to be a universal genetic marker for disease progression in Japanese prostate cancer as well as Swedish.

Renal cell carcinoma: FISH studies on 5q22.3-23.2 demonstrated that gain and loss of 5q22.3-23.2 were predictive genetic marker for favorable and unfavorable patient outcome, respectively in renal cell carcinoma. Cases with 3p loss, the most frequent alteration in renal cell carcinoma, in association with 8q24 (*c-myc*) gain was significantly higher in high stage tumor. Genetic mapping on chromosome 9 using satellite marker showed frequent deletion in *PTCH* gene located on 9q22.

Introduction

Recent advances of molecular biology have revealed that genetic alterations play a crucial role in the oncogenesis and progression of tumors. Thus, it is important for the clinical oncologists to seek genetic markers reflecting the tumor progression, and to select the suitable technique to detect such markers using clinical materials. The fluorescence in situ hybridization (FISH) technique provides information about genetic alterations such as deletion or gene amplification on a cell-by-cell basis regardless of tumor volume, or normal cell contamination (Fig. 1). Hence, this technique has a potential to become a rapid diagnostic tool of the genetic alterations using small tumor volumes.[1),2)] These circumstances prompted the study if the FISH technique could indeed contribute to detect genetic alterations, in particular deletions, reflecting the patient prognosis in urological malignancies.

To localize putative tumor suppressor gene (TSG) has been attempted by loss of heterozygosity (LOH) studies in sporadic tumors, based on the hypothesis that chromosomal deletions unmask a recessive mutation at a specific locus, reflecting the second event of two-hit theory.[3)] Thus, detection of deletion with higher frequency has been used as a way of locating putative TSGs in sporadic tumors. The common chromosomal regions exhibiting LOH with higher frequency were: 3p, 5q, 8p, 11p, 11q, 16 q, 17p and 18q in a variety of malignancies. With regard to chromosome 8p, there is an accumulating evidence suggesting that the putative tumor suppressor genes might be contained in these regions in prostate cancer.[4)] The chromosomal region of 16q, where the E-cadherin gene is located at 16q22, has been supposed to act as TSG in a variety of tumors including hepatocellular, breast, and prostate cancer.[4),5)]

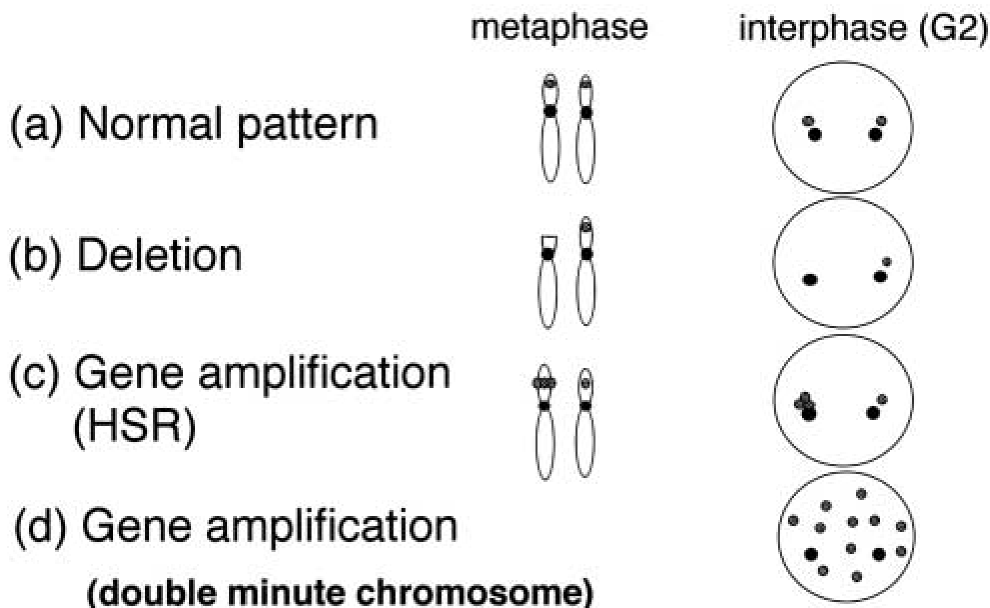

Fig. 1 Chromosomal aberrations detected by FISH
(a) Normal pattern showing two region-specific cosmid spots (half-tone circle) with two centromer spots (closed circle) of the corresponding chromosome.
(b) Deletion pattern showing one region-specific cosmid spots (half-tone circle) with two centromer spots (closed circle) of the corresponding chromosome.
(c) Gene amplification by HSR showing many region-specific cosmid spots (half-tone circle) near the centromer spots (closed circle) of the corresponding chromosome.
(d) Gene amplification by double minute (DM) showing many region-specific cosmid spots (half-tone circle) in the whole nucleus,

The aims of the present study were:

i) To study if the FISH technique could be used as a rapid screening method for the detection of chromosomal aberrations in clinically available cytological samples.
ii) To compare the genomic alterations with clinicopathological parameters.
iii) To explore if the genetic markers could predict disease progression and patient prognosis in urological malignancies

Materials and Methods

Materials: A fresh tumor specimens of bladder, prostate, and renal cell carcinoma (RCC) were obtained by operations both at Department of Urology, Yamaguchi University and Karolinska Hospital, and snap frozen tissues were stored -80 ℃ until use. Totally, 97 patients (41 Japanese and 56 Swedish) with prostate cancer, 90 with transitional cell carcinoma (48 Japanese, 42 Swedish), and 105 with renal cell carcinoma (all Japanese) were studied. The institutional ethical committees approved the studies, and informed consent was obtained from patients.

Methods: Detail of the methods and probe used in these studies were described previously.[6] In brief, digoxigenin-labeled cosmid probes and biotin-labeled centromeric probes of the corresponding chromosomes were co-hybridized on cytological specimens which were made by touch biopsy technique. The hybridization was done at 37℃ overnight in a moist chamber. After adding fluorescent materials, the slides were counterstained with 4', 6-diamidino-2-phenylindole (DAPI), and observed under an epi-fluorescent microscope (Nikon, Tokyo, Japan) through triple band pass filter for DAPI/FITC/trichlororhodamine isothiocyanate (Chroma Technology, Brattleboro, VT) (Fig. 2). Chromosomal numerical aberrations were diagnosed based on centromeric probes as chromosomal losses (monosomy) or gains (trisomy or tetrasomy) when the percentage of nuclei with one, three or four signals, respectively, exceeded 10%.[7] Chromosomal deletion was defined as when the fraction of nuclei with a lower number of cosmid signals than centromeric signals (decreased fraction) exceeded 35% out of over 150 nuclei counted by two independent observers.[6]

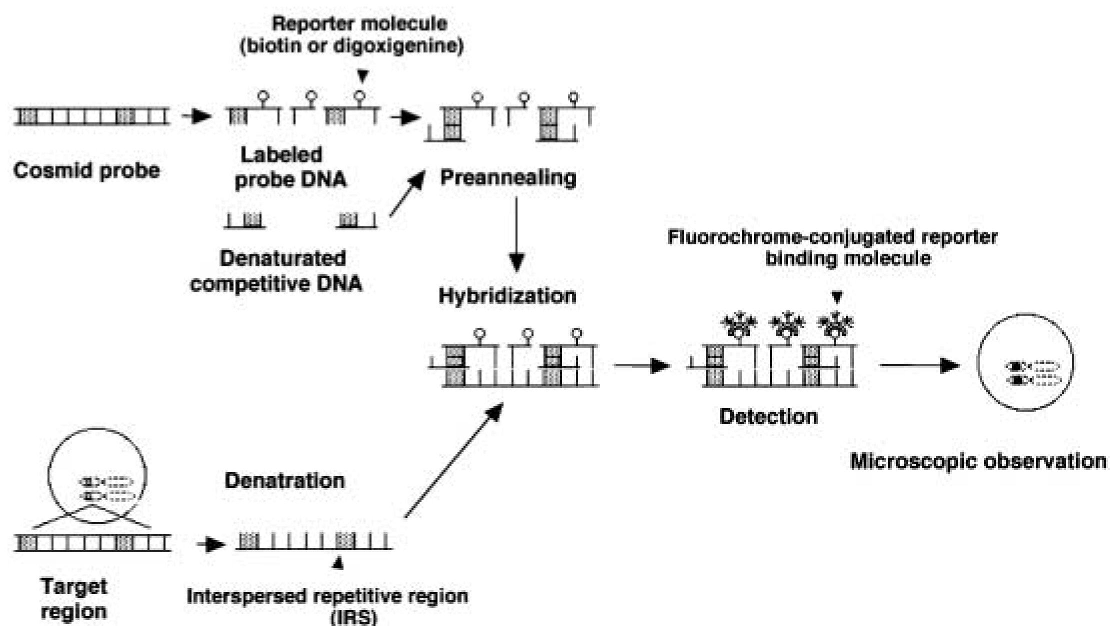

Fig. 2 Schema of fluorescence in situ hybridization technique.

Results

Numerical aberrations of chromosomes 7, 9, 10, and its clinical significance in bladder cancer

Out of 33 evaluable Swedish cases, 29 (88%) demonstrated either chromosomal aberrations of chromosomes 7, 9, 10 or 11 in DNA-diploid bladder cancer. In particular, trisomy 7, monosomy 9 and trisomy 10 seemed to be non-random chromosomal aberrations which might be early events in the evolution of bladder cancer (Fig. 3).[7] Additionally, 48 consecutive cases of Japanese bladder cancer were studied using slides which had been applied for ordinal urine cytological diagnosis. Chromosomal aberrations were detected in 9 of 18 patients with negative or equivocal cytology by the Papanicolaou classification. In conclusion, monosomy of chromosome 9 might be a prognostic marker for early tumor recurrence in patients with negative or equivocal cytology specimens.[8]

p53 deletion and nuclear p53 overexpression as a genetic marker for tumor recurrence and tumor progression in bladder cancer.

*p*53 deletion and nuclear p53 overexpression were studied in 42 (Swedish), and 30 (Japanese) specimens of transitional cell carcinoma, respectively. *p53* deletion was demonstrated in 64% of specimens. The *p*53 deletion was significantly correlated with grade ($p<0.01$), stage ($p<0.05$), S-phase fraction ($p<0.05$), and DNA ploidy ($p<0.01$).[9] Patients with p53 overexpression had significantly higher tumor progression rate, and

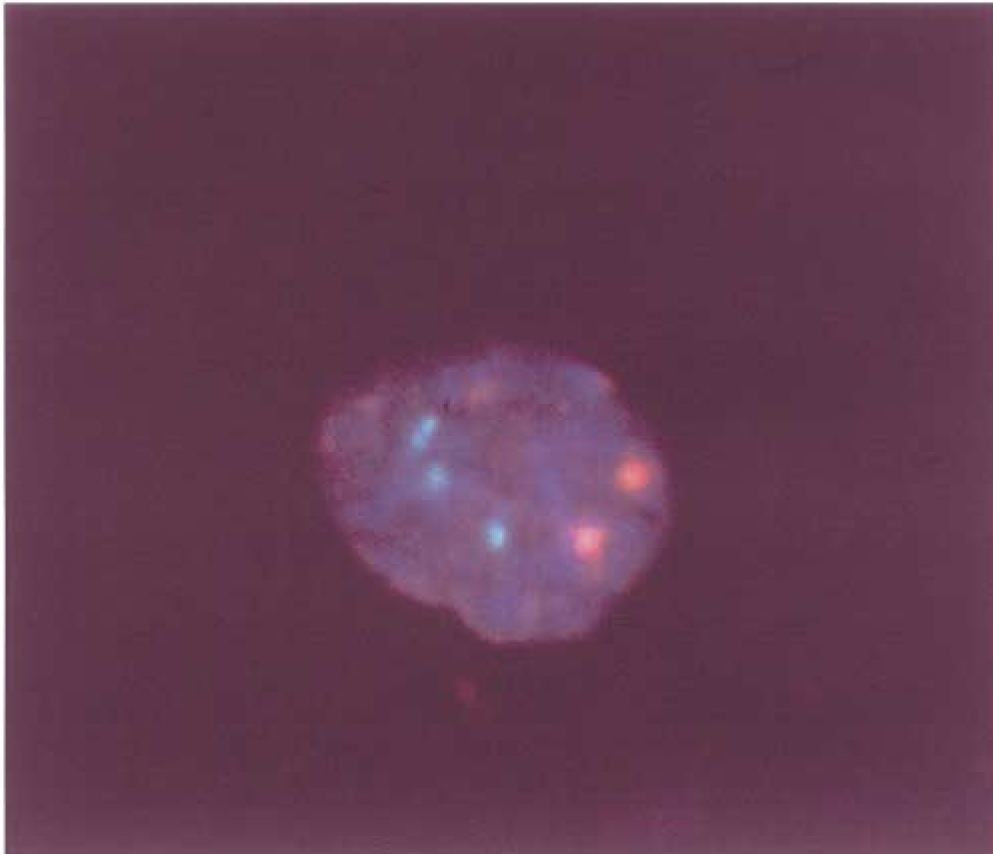

Fig. 3 Representative case of trisomy 7.
Three green spots showing centromer part of chromosome 7 mean three copy number of chromosome 7 (trisomy). Note two red spots showing centromer part of chromosome 9 mean two copy number of chromosome 9 (disomy, normal pattern).

lower recurrence-free survival than those without overexpression.[10] These results suggest clinical significance of p53 deletion and nuclear p53 overexpression as genetic marker for assessing both malignant potential and patient outcome in human urothelial tumors.

The role of chromosome deletions of 8p and 16 q in prostate cancer

Chromosome deletions on 8p loci (8p12, 8p 21.1-21.2, 8p21.3, 8p22 and 8p23-pter), 10 q24-qter, and 16q24 were studied in prostate cancer (Fig. 4).[6),11)−16)] In total, several types of 8p deletions were noted in 71% of the patients.[6)] Deletions spanning 8p21-22 were significantly correlated with tumor grade and stage.[13)] In particular, Cox's proportional hazard model reveled 8p22 deletion to be the strongest parameter to predict disease progression (hazard ratio=5.75; p=0.0025).[14)] Patients whose tumors showed both 8p22 and 16q24 deletions had a significantly higher frequency of nodal metastases than non-metastases.[12),15)] These data suggest that estimation of 8p22 and 16q24 deletions may serve as a genetic diagnosis for predicting pathological staging as well as disease progression in prostate cancer.

Links between genetic and environmental factors and prostate cancer risk

In order to explore the racial genetic background regarding susceptibility on prostate cancer risk, genetic polymorphisms of repeat number of CAG repeat in exon 1 of the androgen receptor were studied, and compared among 33 Japanese and 59 sporadic, 59 hereditary Swedish prostate cancer patients.[17),18)] Both Swedish sporadic and hereditary prostate cancer had shorter CAG repeats than Swedish controls, while Japanese prostate cancer patients had longer repeat than control. These differences between two populations suggest that two populations have different genetic background regarding CAG repeats, and that CAG repeats are

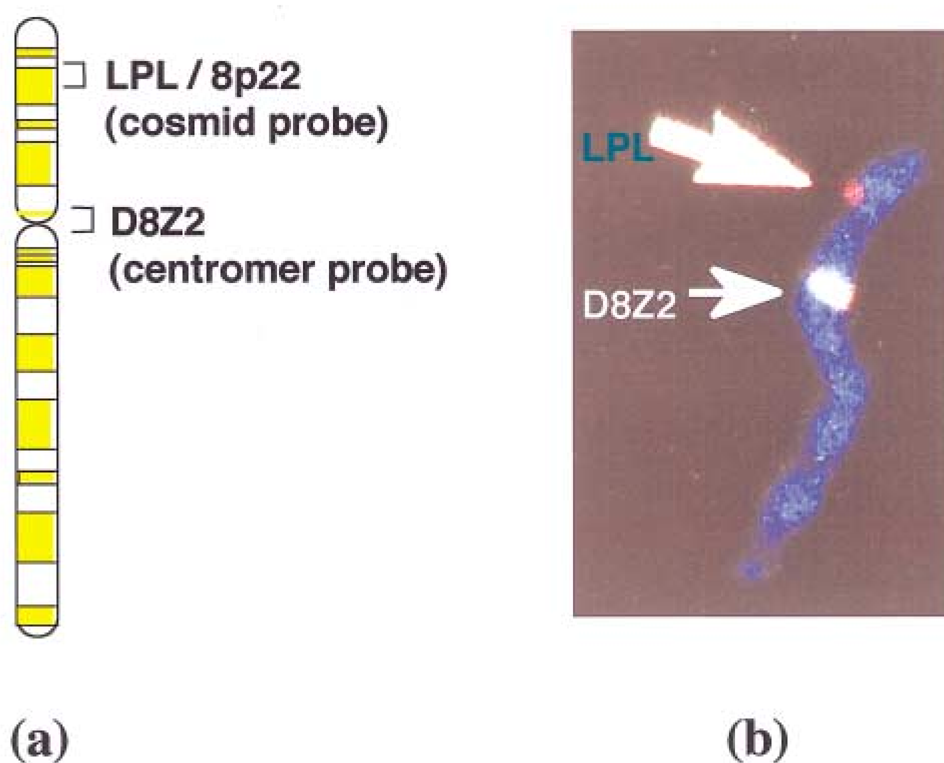

Fig. 4 Schematic (a) and actual mapping position (b) of LPL /8p22 (cosmid probe) and D8Z2 (centromer probe) of chromosome 8.
Note LPL cosmid probe (red spot) is correctly hybridized to 8p22 region on the metaphase chromosome.

in linkage disequilibrium with a prostate cancer susceptibility gene locus flanking or overlapping the AR gene at xq12.1.

Clinical significance of allelic loss of 5q22.3-23.2, and 3p25 with c-myc gain in renal cell carcinoma

Deletion mapping on chromosome 5q spanning 5q21 to q23, and chromosome 9 spanning 9p21 to 9q34.3 were analyzed in over 100 patients with renal cell carcinoma (all Japanese), by FISH technique[19], and fluorescent multiplex-polymerase chain reaction technique[20], respectively. Further study on association of allelic loss of 3p25 including *VHL* gene with chromosome 8p loss and 8q24 (*c-myc*) gain was carried out in 50 Japanese renal cell carcinomas (RCC).[21] Alterations of chromosome 5q22.3-q23.2 could be a useful genetic marker for predicting patient prognosis of RCC. Results from chromosome 9 and 3p25 studies demonstrated the conclusion that loss of heterozygosity of the *PTCH* gene (9q22) and allelic loss of 3p25.1-25.3 with *c-myc* gain are related to the development of RCC.

Discussion

There is accumulating evidence that chromosomal numerical aberrations lead to chromosomal instability that confers tumors to higher malignant potential. Monosomy 9 was found in grossly normal bladder mucosa from patients with bladder cancer as well as in the corresponding tumor. The result may indicate that monosomy 9 is one of the field defects in bladder cancer.[22] The concept of a field defect means a common genetic alteration in the tumor as well as its adjacent normal tissue. Non-random genetic aberrations due to some initiators and subsequent chromosomal instability occurring within the precancerous cells seems to be essential for the oncogenesis of bladder cancer.[23] Frequent deletions of chromosome 9pq or monosomy 9 suggest that these alterations may occur as early events in bladder cancer, hence these alterations may be useful for powerful genetic diagnostic tool for detecting tumor recurrence.

Regarding genetic alterations of prostate cancer, there is evidence for two, or possibly three TSG's on chromosome 8p. We have demonstrated a high frequency of deletions on 8p21 (*NEFL* locus) and a possible break point between 8p21 and 8p12, leading to the involvement of both 8p22 and 8p21.3 as a minimal deleted region[4),13)] Recent functional analysis has demonstrated that LZTS 1 (*FEZ1*) gene at 8p22 is involved in the regulation of cell growth[24], and its loss of function may contribute to the development of prostate cancer. Genetic pattern seems to be similar between Japanese and Caucasian prostate cancers in terms of 8p deletion.[5),14)] In contrast, genetic polymorphism of CAG repeat was completely different between Japanese and Swedish. This discrepancy may be explained by that different genetic polymorphism of androgen receptor including CAG repeat, or flanking unknown region reflects different susceptibility of clinically manifested prostate cancer between two races. Whereas 8p22 deletion may be a common alteration in sporadic prostate cancer irrespective of different races probably due to late genetic event. 8p22 deletion could be a universal genetic marker for predicting disease progression.

An increased copy number of 5q22-23 may result from the early genetic event, while allelic loss of these regions may be a late genetic event, since cases with the 5q22-23 gain had significantly favorable prognosis than those with loss of these regions. Putative tumor suppressor gene may be involved in these regions. The *c-myc* gene, mapped to chromosome 8q24 has been reported to link to growth regulation, cell differentiation, and apoptosis. Interestingly, cases with both c-myc gain and 3p deletion had significantly larger tumor sizes than those that did not. However, there was no relationship in tumor grade, or size when *c-myc* gene alone was compared to such parameters. Based on these results, it is likely that translocation involving chromosome 3 and 8 may confer higher malignant potential regarding tumor development in RCC.

In conclusion, identification of genetic alterations of the specific chromosomal region may realize the genetic diagnosis for detect-

ing early stage cancer, or tumor progression in urological malignancies.[25] Further study will confirm the reality of routine clinical application of the technique in future.

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