Sex Chromosome Alterations Associate with Tumor Progression in Sporadic Colorectal Carcinomas

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Abstract

Purpose: The X and Y chromosomes have been associated with malignancy in different types of human tumors. This study attempts to determine the involvement of X chromosome and pseudoautosomal regions (PAR) in sporadic colorectal carcinogenesis.

Experimental Design: An allelotyping of X chromosome in 20 premalignant and 22 malignant sporadic colorectal tumors (CRC) from female patients and an analysis of losses [loss of heterozygosity (LOH)] on PARs from 44 CRCs and 12 adenomas of male patients were carried out. In male tumors, a fluorescence *in situ* hybridization analysis was done to identify which sex chromosome was possibly lost.

Results: The LOH frequency in female CRCs was 46% with higher incidence in patients with tumor recurrence than in those who were disease-free ($P < 0.01$) and with a significant difference from adenomas (11%; $P < 0.0001$). The LOH rate of PARs in male CRCs was 37% with a frequency significantly higher in patients with recurrence ($P < 0.03$). These results were maintained also when data from PARs of all 66 male and female patients were cumulated ($P < 0.05$). LOH in PARs was significantly correlated with LOH at 5q ($P < 0.01$) and 18q ($P < 0.01$), early and late events, respectively, in colorectal carcinogenesis. Fluorescence *in situ* hybridization analysis in male patients with extensive PAR LOH revealed a preferential loss of the Y chromosome.

Conclusions: Our data suggest a role for sex chromosome deletions in the malignant progression of sporadic CRCs and support the presence in the PARs of putative tumor suppressor genes involved in the progression of human sporadic CRCs.

The human X and Y chromosomes are morphologically and genetically distinct. The X chromosome is large and euchromatic and contains almost 1,000 genes (1), whereas the Y chromosome is small and heterochromatic and contains at least 160 genes (2, 3). A number of genes retain copies on both X and Y chromosomes and escape the inactivation that affects most X-linked genes in somatic cells (4). At both ends of sex chromosomes, X-Y homologous regions are present: these are pseudoautosomal regions (PAR) that pair regularly at male meiosis and undergo recombination (5). The unique biology of these regions has attracted considerable interest because of the identification of new tumor suppressor genes whose alterations may play a role in the development and progression of human tumors.

X chromosome was found to be involved in carcinogenesis and the malignant progression of different types of human tumors, and an increasing number of potentially responsible genes have been identified (1). In particular, gains or deletions have been associated with tumoral progression, the presence of metastases, and worse prognosis in tumors of the breast (6), ovary (7), and uterine cervix (8) and in papillary renal cell carcinomas (9). With regard to Y chromosome, deletions have been shown to be involved in prostate cancer (10–14), male breast carcinomas (15–17), and pancreatic adenocarcinomas (18).

In previous studies done in our laboratory on neuroendocrine tumors, we have shown a high frequency of allelic losses [loss of heterozygosity (LOH)] in endocrine carcinomas of the gastroenteropancreatic tract (19–21) and of the lung (22). In all these tumors, the LOH rate was associated with malignancy and poor prognosis (19–21). These data were in accordance with the study of Missiaglia et al. (23) who showed a strong association between X and Y chromosome alterations and aggressiveness and shorter survival rates in nonfunctioning pancreatic endocrine tumors.

On these bases, we extended the study of X chromosome deletions to nonendocrine colorectal tumorigenesis. Our aim was to verify the involvement of X chromosome and of PARs of X and Y chromosomes in a well-characterized tumor model of the digestive tract in which a multistep and multipathway carcinogenesis can be followed all the way from noninvasive

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premalignant lesions (i.e., adenoma) to invasive cancer accompanied by accumulation of specific genetic alterations (24, 25).

We carried out an extensive allelotyping of X chromosome in premalignant and malignant sporadic colorectal lesions of female patients, whereas in male patients an analysis for LOH on PARs of X and Y chromosomes was carried out. Moreover, a fluorescence *in situ* hybridization (FISH) analysis was done in tumors of male subjects to identify which sex chromosome, X or Y, was involved in the losses. Finally, the relation of X or Y LOH with alterations of gene regions commonly involved in colorectal carcinogenesis such as 5q, 8p, 4p, 18q, and p53 was investigated.

Materials and Methods

Patients. For X chromosome allelotyping, 22 primary sporadic colorectal adenocarcinomas of female patients were studied. Of these tumors, 10 were from patients free of disease 3 years after surgery (age range, 53-91 years; mean, 70 years) and 12 were from patients with recurrence of disease after surgery (age range, 48-86 years; mean, 68 years). The latter included three patients in which metastatic tissue was removed together with the primary tumors. For the study of PAR, we analyzed 44 sporadic colorectal tumors (CRC) from male patients, 29 disease-free (age range, 33-94 years; mean, 76 years) and 15 with recurrences, 3 years after surgery (age range, 62-88 years; mean, 74 years). No case of palliative surgery was inserted in this study. In both groups, cases with microsatellite instability were excluded. In accordance with the classification of tumors by the WHO (26), tumors were defined as mucinous when ≥50% of the tumor mass consisted of accumulated mucin (mostly extracellular) or were classified as "adenocarcinoma, not otherwise specified (NOS)". Tumors were staged in accordance with the tumor-node-metastasis system (27). Moreover, proximal colon (right) was defined as the large bowel proximal to the splenic flexure, and distal (left) colon was defined as the large bowel distal to the splenic flexure. Patients were observed at 3-month intervals for 24 months after the completion of therapy, then every 6 months for 3 years, and then yearly. All patients of this study received the same adjuvant therapy as defined by Moertel et al. (28). The drug regimen for chemotherapy was 375 mg/m^2/d 5-fluorouracil and 20 mg/m^2/d levamisole, 5 days/wk every 4 weeks for 6 months.

For the study of premalignant conditions, 32 colorectal adenomas, all obtained at endoscopy, 20 from female patients (age range, 51-91 years; mean, 71 years) and 12 from male patients (age range, 40-79 years; mean, 64 years), with different histologic structure and grades of dysplasia, were analyzed.

Tissue specimens. Matched tumor and adjacent normal tissue specimens of freshly resected colorectal carcinomas were snap frozen in liquid nitrogen and subsequently stored at -80°C, whereas all the adenomas were investigated on routinely formalin-fixed paraffin-embedded tissues.

Immunohistochemistry and semiquantitative analysis of p53 protein. Five-micron sections were stained with H&E for histologic diagnosis and with the primary monoclonal antibody anti-p53 (clone DO7, DAKO; working dilution of 1:50). For antigen retrieval, sections were treated with 10 mmol/L citrate at pH 6.0 in a 750-W microwave oven for three 5-min cycles. The sections were immunostained with the streptavidin-biotin kit (LSAB2, DAKO) in accordance with the manufacturer's specifications and counterstained with hematoxylin. Negative controls consisted of substituting normal mouse serum for the primary antibodies.

The expression of p53 was analyzed on the basis of the frequency of positive cells using a cutoff level of 10% and the cases subdivided into three classes, as previously proposed (29–31): nonexpressing, expressing up to 10% of immunoreactive cells, and overexpressing (>10%).

Table 1. Microsatellite markers used in the study and cytogenetic location

Microsatellite markers	Cytogenetic band
DXYS233	Xp22.32-Yp11.3
SHOX	Xp22.32-Yp11.3
DXS996	Xp22.3-p22.3
DXS207	Xp22.2-p22.2
DXS989	Xp22.13
DXS1237	Xp21.3-p21.2
MAOA.PCR1	Xp11.4-p11.3
DXS1003	Xp11.23
DXS1367	Xp11.3-p11.23
Centromere	
DXS1111	Xq12
DXS56	Xq13.2-q13.3
DYS233	Xp22.32-Yp11.3
DXS738	Xq21.1-q21.1
DXS990	Xq21.33
DXS1153	Xq22.1-q22.3
DXS1220	Xq22.3-q23
DXS1001	Xq24
DXS1047	Xq25-q26
DXS102	Xq26
DXS1192	Xq26
DXS731	Xq27-q28
DXYS154	Xqter-Yqter

DNA extraction and PCR. DNA was extracted using the Qiagen DNeasy tissue kit from 15 to 25 cryostat sections (20 μm thick) of the tumors and matching normal samples and from 5-μm sections stained with hematoxylin for adenomas. Only tumor samples containing at least 80% of neoplastic cells were included in the study.

The tissues derived from female patients were allelotyped for 21 polymorphic microsatellite markers covering the whole X chromosome whereas those from male patients for the three markers localized in the pseudoautosomal regions PAR I and PAR II common for X and Y chromosomes (Table 1). The influence of formalin fixation and paraffin embedding on the results of the study was tested by parallel analysis of all 21 microsatellite markers in both frozen and fixed tissues obtained from the very same colorectal cancer in six female patients.

CRCs were previously analyzed for LOH on chromosomes 4p, 8p, and 18q (32) and for 5q in the locus D5S346 mapping for *APC* gene.

The markers were selected from the Genome database[3] on the basis of chromosomal location. Forward primers were synthesized with a fluorescent tag (WellRed dyes from Research Genetics). DNA was amplified in a 25-μL reaction solution containing 2.5 μL of 10× buffer (Promega), 1 to 2 mmol/L $MgCl_2$, 0.4 μmol/l primer pairs, 200 μmol/L deoxynucleotide triphosphates, and 1.25 units of Taq polymerase (Promega). Amplifications were done using a 5-min initial denaturation at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 53°C to 64°C, and 1 min at 72°C; or by 40 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The fluorescently labeled PCR products were subjected to electrophoresis on an automated DNA sequencer CEQ 2000XL (Beckman Coulter, Inc.), and the fluorescent signals from the different sized alleles were recorded and analyzed using CEQ 2000XL analysis software (Beckman Coulter).

Definition of allelic loss. The allelic losses were defined as the ratio of relative allelic peak height in the tumor DNA to relative allelic peak height in the corresponding normal DNA. The formula used for the calculation was T2:T1/N2:N1, where T1 and N1 are the height values for the smaller allele and T2 and N2 are the height values for the larger

[3] http://www.gdb.org and http://www.geneatlas.org

allele of the tumor (T) and normal (N) samples, respectively. For informative markers, LOH was scored when signal reduction for one allele was ≥40% (33).

FISH. Nine male patients with extensive PAR deletions were used for FISH evaluation. For the analysis, 3-μm sections of formalin-fixed paraffin-embedded tumor tissue were used and areas suitable for hybridization were identified by microscopic examination of consecutive H&E-stained sections. The chromosome enumeration probe (CEP) was a mixture of a spectrum orange–labeled CEP X probe specific for the α satellite centromeric region of chromosome X and a spectrum green–labeled CEP Y probe specific for the satellite III (Yq 12) region of chromosome Y (Vysis, Inc.). Target DNA on the slides and the probe mixture were denatured together (5 min at 85°C) and hybridized overnight at 37°C using the Hybrite system (Vysis). Posthybridization wash was applied (20% SSC/NP40) for 3 min at 72°C. The slides were then counterstained using 4′,6-diamino-2-phenylindole and processed with an Olympus BX60 fluorescence microscope equipped with a 100-W mercury lamp. Separate band pass filters were used for the detection of CEP X orange spectrum and CEP Y green spectrum probe signals. After 4′,6-diamidino-2-phenylindole counterstaining, fluorochrome signals were captured individually and images were generated via computer using Quips genetic Workstation and Imaging Software (CytoVision). The absence of orange or green spots in the tumoral component was indicative for chromosome X or Y losses, respectively.

Statistics. Data were examined with χ^2 analysis and Fisher's exact test corrected for multiple testing. These analyses were done using the SPSS 11.5 statistical software package (SPSS, Inc.). Spearman rank correlation test was used to assess the relationship between clinicopathologic and molecular variables and LOH in at least three loci on X chromosome or in at least one locus on PARs. Moreover, Spearman rank correlation test was used to verify if the LOH condition in the PARs was representative of the remaining portions of the X chromosome for each female patients. To this end, the data were categorized on the basis of the frequency of LOH for each patient: 0, no LOH; 1, one LOH in the PARs, one to three LOH on the remaining X chromosome; 2, more than two LOH in the PAR region, more than three for the remaining X chromosome. Two-sided $P < 0.05$ was considered statistically significant.

Results

Allelotyping of X chromosome. The results of the allelotyping of 21 microsatellite markers spanning the whole X chromosome and the degree of informativeness in 22 CRCs of female patients are shown in Figs. 1A and 2. The 22 CRCs of the female patients analyzed in this study showed a degree of informativeness (i.e., the percentage of informative loci from the whole series of cases) for the 21 microsatellite markers of 73% (27-96%) with an LOH rate ranging from 29% (*DXS1237*) to 68% (13 of 19) at *DXS1047* locus (Fig. 1A). Moreover, the frequency of X chromosome LOH was 46%, with a statistically significant difference ($P < 0.01$) between patients with recurrent disease (53%) and those who were disease-free (39%). Deletions of at least one site were found in 19 (86%) cases and of more than three sites in 13 (59%) cases. Of the latter, the allelic losses were continuous in 8 (36%) CRCs, suggesting loss of the entire X chromosome (Fig. 2). The LOH frequency was higher in tumors localized in proximal colon (60%; $P < 0.012$), whereas no relations were found with age, stage, and histotype (mucinous versus NOS).

In the 20 colorectal adenomas of female patients, the degree of informativeness was 71% (50-94%). The LOH frequency was 11%, significantly lower than that of CRCs ($P < 0.0001$). No differences were found between cases with mild dysplasia (8%) and those with moderate or severe dysplasia (12%) or between tubular (8%) and villous or tubular-villous (10%) histotypes.

The comparative analysis of snap-frozen and formalin-fixed paraffin-embedded tissues in the very same cancer from six female patients showed a very similar pattern of LOH with the 92% of concordant results, thus supporting our comparison of data from adenomas with those from CRCs.

Frequency of LOH in PARs. The LOH on three microsatellite loci (*DXS154*, *SHOX*, and *DXS233*) localized in the PARs was analyzed in 44 CRCs from male patients, and the degree of informativeness was 75% for *DXYS233* and *SHOX* and 89% for *DXYS154* (Fig. 1B). The LOH frequency was 37% (39 of 106) with a statistically significant difference ($P < 0.03$) between patients with recurrent disease (49%; 19 of 39) and those who were disease-free (30%; 20 of 67). These results were maintained when all 66 CRCs from both male and female patients were cumulated, with a LOH frequency of 39% (62 of 158) and a higher incidence ($P < 0.05$) in patients with recurrent disease (51%; 33 of 65) than in those who were disease-free (31%; 29 of 93). The latter result was also observed when the number of patients showing at least one LOH in the PAR was considered ($P < 0.03$). The LOH rate did not correlate with sex, site, age, stage, and histotype (mucinous versus NOS). Taking all 66 CRCs together, the most frequently deleted marker was *DXYS233* (51%), an incidence significantly higher ($P < 0.05$) than that of *SHOX* (27%) and *DXYS154* (39%). The LOH frequency at *DXYS233* tended to be higher in patients with recurrence of disease (62%) than in those who were disease-free (41%), although not statistically significant. In the 32 adenomas, the frequency of PAR LOH was significantly lower (18%) than in CRCs ($P < 0.01$). No differences were found between cases with mild dysplasia (10.5%) and those with moderate or severe dysplasia (26%) or between tubular (23%) and villous or tubular-villous (no LOH) histotypes. The LOH rates in the PARs

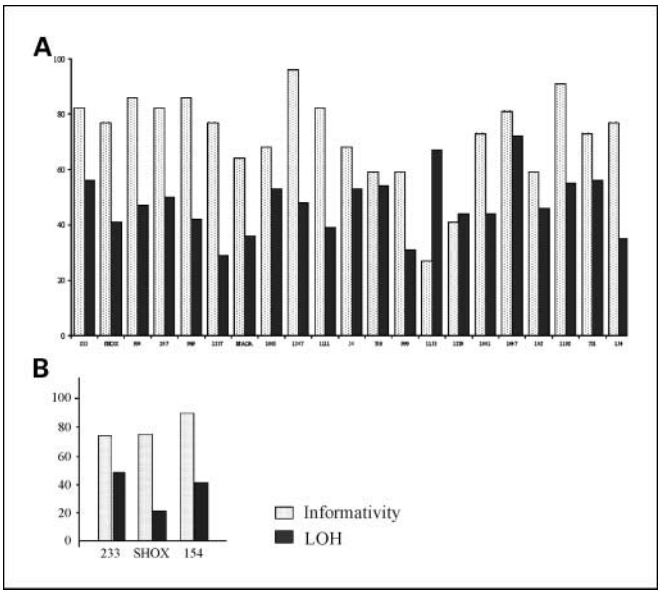

Fig. 1. *A*, degree of informativity and LOH rate of 21 microsatellite markers spanning the whole X chromosome in 22 CRCs of female patients. The LOH rate was calculated as percentage of informative cases. *B*, degree of informativity and LOH rate in three markers of the PARs in 44 CRCs of male patients. The LOH rate was calculated as percentage of informative cases.

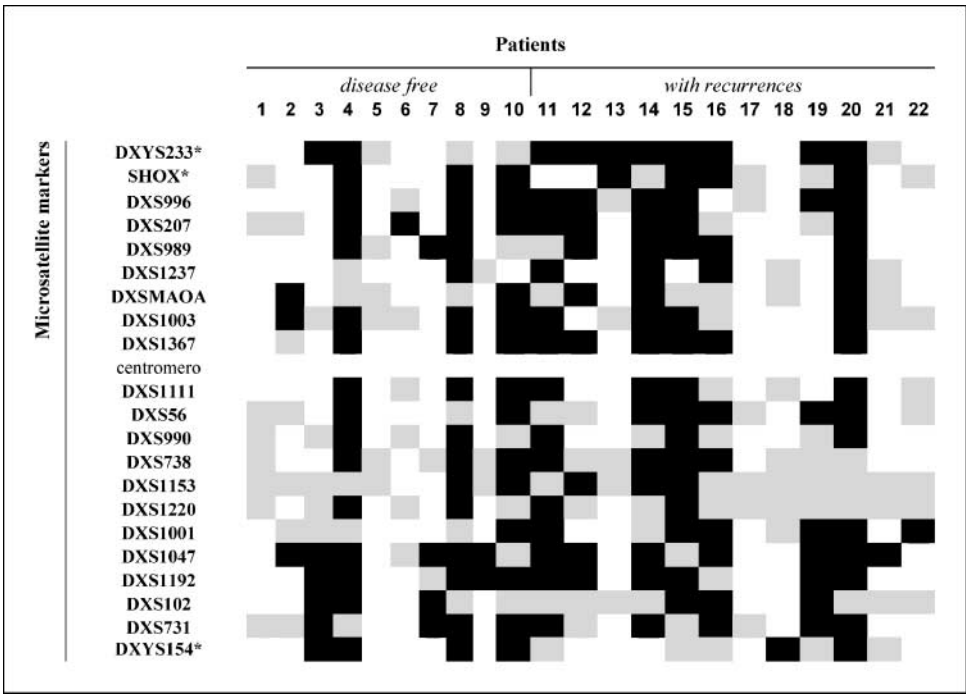

Fig. 2. Allelotyping results in colorectal carcinomas from 22 female patients. ■, LOH; □, retention of heterozygosity; ▩, noninformative, *, PARs.

and in X chromosome from 22 female patients were 44% and 47%, respectively, with a statistically significant correlation ($P < 0.0019$; $r = 0.62$), indicating that the LOH condition of the PARs may reflect that in the remaining portions of the X chromosome.

FISH Data. At FISH analysis, 7 of 9 (78%) CRCs from male patients with multiple LOH on PARs also showed lack of the green signal for the CEP Y probe, indicating the complete loss of Y chromosome (Fig. 3A). As a control labeling, both CEP Y and CEP X probes were seen in the peritumoral normal mucosa (Fig. 3B).

Chromosome X and PAR LOH versus 18q, 8p, 4p, 5q LOH and p53 overexpression. None of 18q, 8p, 4p, 5q, p53 alterations correlated with the LOH rate on chromosome X in female patients even if the analysis was restricted to tumors with at least three loci lost. Instead, the LOH rate in PAR markers of all 66 CRCs correlated with that on chromosome 18q ($P < 0.03$; $r = 0.27$). All seven cases with LOH in three markers of the PARs showed LOH in both markers of chromosome 18. Allelic losses on at least one of the three PAR loci correlated with the presence of LOH on 5q using markers strictly associated with *APC* gene ($P < 0.03$; $r = 0.26$). No significant correlations were found between PAR LOH frequency and chromosomes 8p and 4p. p53 immunohistochemistry of 62 tumors showed 22 cases fully unreactive (33%), 10 cases with <10% immunostaining (15%), and 30 overexpressing (>10%) cases (45%). No significant correlations were found between the PAR LOH frequency and the immunohistochemical expression of p53 (Fig. 3C).

Discussion

X chromosome alterations have been found in neoplasms of different organs. In particular, loss of X chromosome, with a frequency ranging from 20% to 30%, has been reported by cytogenetic analysis for a wide variety of tumors including those of lung, ovary (34, 35), testis, and nervous system, as well as melanomas (34). In our laboratory, X chromosome LOH has been investigated in neuroendocrine carcinomas of the gastroenteropancreatic tract (19–21) and of the lung (22), showing a strong association with malignancy and poor prognosis, and suggesting the involvement of X chromosome oncosuppressor gene(s) in tumor progression.

To date, little is known about the involvement of X chromosome in CRCs, with the exception of one cytogenetic study of 79 CRCs showing the involvement of sex chromosomes in the CRC carcinogenetic process (36) and of a comparative genomic hybridization study showing a high level of gains on X chromosome in a series of 45 sporadic CRC (37). In the present study, the allelotyping analysis using 21 markers on X chromosome in 22 sporadic CRCs from female patients showed a LOH frequency of 46% with a significantly higher incidence in patients with tumor recurrence than in those who were disease-free. The relation of X chromosome LOH with malignant progression was further emphasized by our results in colorectal adenomas, which showed a significantly lower frequency of X chromosome LOH (11%) as compared with CRCs. This finding suggests that X chromosome LOH is a late event in colorectal tumorigenesis and is in agreement with previous data about tumors of the breast (6), ovary (7), uterine cervix (8), and papillary renal cell carcinomas (9) showing association between losses on X chromosome and tumoral progression, presence of metastases, and worse prognosis. Of interest, in nine cases of CRCs we found X chromosome deletions in virtually all microsatellite markers analyzed, suggesting the loss of the entire X chromosome.

To verify the further involvement of sex chromosomes in CRC carcinogenesis in male patients, we carried out an LOH analysis of PARs, the PARs homologous to X and Y chromosome regions.

In this regard, the comparison of the PAR LOH and the LOH status of the remaining sex chromosome in CRCs of female patients revealed that the LOH rate in the PAR is related to that of the whole X chromosome.

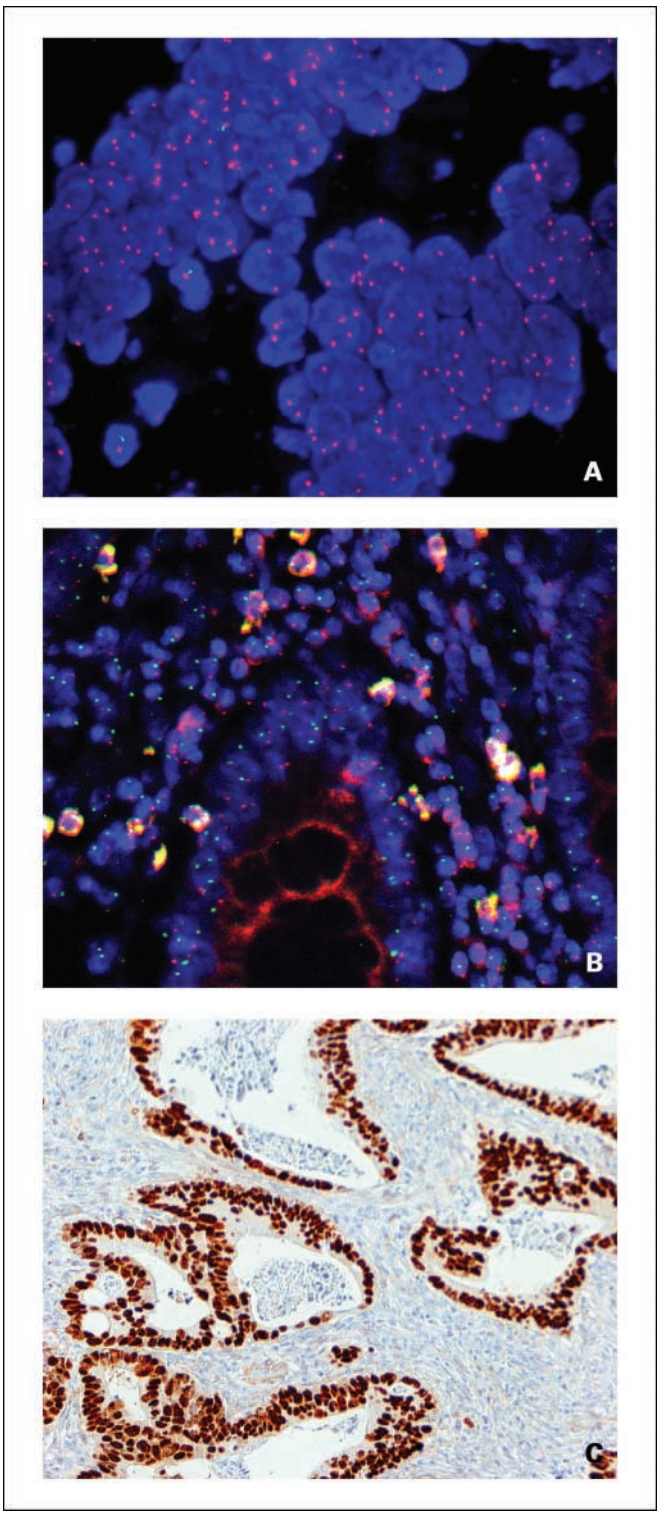

Fig. 3. Colorectal carcinoma from a male patient. *A,* absence of green signal (CEP Y) in the tumoral cells indicating the complete loss of Y chromosome. *B,* presence of both green (CEP Y) and red (CEP X) spots in the control normal mucosa. *C,* representative finding of intense immunohistochemical expression of p53 protein in a colorectal carcinoma.

Our results with the LOH analysis of the PARs in CRCs of male patients were consistent with those obtained with the LOH analysis of the whole X chromosome in CRCs of female patients. The LOH rate was 37% and the incidence in patients with tumor recurrence was significantly higher than in those who were disease-free at 3-year interval. Moreover, the PAR LOH rate of adenomas was lower than that of CRCs. These results were maintained also when data from PARs of all 66 male and female patients were cumulated.

Interestingly, the LOH rate in the PARs showed a significant correlation with the occurrence of LOH at 5q, in a locus strictly associated with *APC* gene, and at 18q, which, in the colorectal carcinogenic sequence, are known to be early and late events, respectively (38, 39). The association with a late event such as 18q LOH suggests that PAR deletions are involved in the late carcinogenic steps of sporadic CRCs. On the contrary, the lack of correlation of the LOH rate in PARs either with losses in 8p and 4p or with age is against the possibility that PAR LOH depend on a more generalized chromosomal instability or are an effect of aging.

To verify which sex chromosome was involved in the deletions shown by LOH analysis in the PARs, a FISH analysis done in CRCs of nine male patients with extensive PAR LOH consistently revealed lack of stain for the Y CEP, indicating loss of the whole Y chromosome in these patients.

Several observations suggest that genes located in PARs may play some role in controlling cell proliferation. Eight human pseudoautosomal genes have been described thus far in the Xp/Yp PAR I region (*MIC2*, *XE7*, *ASMT*, *ANT3*, *IL3RA*, *CSF2RA*, *SHOX*, and *PGPL*; ref. 5) but only two in the Xq/Yq PAR II region (*SYBL1* and *IL9R*; refs. 40–42). *MIC2* was the first pseudoautosomal gene described in man and is a ubiquitously expressed housekeeping gene that encodes a cell-surface antigen (43). *XE7* seems to be ubiquitously expressed, with alternative splicing in two very hydrophilic protein isoforms with unknown biological function (44). The *ASMT* gene has been suggested as a candidate for psychiatric disorders due to its brain and retina expression and association with schizophrenia in linkage studies (45, 46). *ANT3* is a highly conserved gene from the ADP/ATP translocase family playing a fundamental role in the energy metabolism of the eukaryotic cell (47). *IL3RA* and *CSF2RA* map closely and their products share the same β subunit whereas the (48) subunits are distinct (48, 49). *SHOX* and *PHOG* genes are suggested as causing growth failure in idiopathic short stature and Turner syndrome (50, 51). The high degree of conservation across several species of the *PGPL* gene implies an important function for the locus in humans (52). Its expression pattern as well as the important biological role carried out by the encoded GTP-binding protein suggests that the gene is crucially involved in the vitality of an organism (52).

In conclusion, our data suggest a role for sex chromosome losses in the malignant progression of sporadic CRCs. Moreover, our results support the evidence of target regions for LOH in the PARs, suggesting the presence of one or more putative tumor suppressor genes that may play a role in the development and progression of human sporadic colorectal cancer.

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