SPANDIDOS PUBLICATIONS

Allelic loss of Hox11L1 gene locus predicts outcome of gastrointestinal stromal tumors

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Abstract. Loss of heterozygosity (LOH) in tumors has been described to have prognostic impact. Hox11L1 gene, located on chromosome 2, has a role in proliferation of neuronal myenteric Cajal cells being the progenitor cells of GISTs. The aim was to examine the frequency and prognostic value of allelic loss of Hox11L1 gene locus in GISTs. Tumor and control DNA of 72 GIST patients was extracted after microdissection from tissue sections. Patients underwent surgery between 1992 and 2003 and were histopathologically reclassified. Microsatellite marker D2S286 on chromosomes 2 near Hox11L1 gene locus was used for detection of LOH by PCR and capillary electrophoresis. Survival was calculated by Kaplan-Meier plots. LOH was found in 7 (10%) of 72 GISTs. Fifty-four (75%) cases did not show LOH. Eleven (15%) were homozygous and consequently non-informative. Survival analysis (n=59) revealed a significantly worse tumor-specific and relapse-free survival for GIST patients with LOH in the tumor by univariate analysis ($p<0.05$ by log-rank test; median follow-up time 37 months). LOH of Hox11L1 gene locus is a useful parameter for prognosis of GIST. The data propose that Hox11L1 has a role in tumorigenesis in GISTs.

Introduction

Gastrointestinal stromal tumors (GISTs) are a heterogeneous group of tumors originating from the interstitial cells of *Cajal* that are located in the nerve plexus of the muscularis in the gut wall (1). On primary manifestation the outcome of GISTs is hardly predictable and patients have a poor prognosis once relapse or metastasis is discovered (2,3). The remarkable anti-tumor activity of the protein kinase inhibitor Imatinib (Glivec®) in GISTs requires accurate diagnosis (4). Most GISTs occur in the stomach and small intestine, but rarely they also present in the esophagus and large intestine (5). Several classifications of GISTs exist, one of them is the 'consensus approach of 2002' that considers primary tumor size and mitotic rate (1). The outcome clearly depends on these two parameters (6). Tyrosine kinase *c-kit* (CD117) and PDGFR play important roles for diagnosis and therapy of GISTs (1,4). A definitive discrimination between benign and malignant GISTs, however, has not been achieved and the mechanisms of tumorigenesis are unknown.

Genetic instability exists at two distinct levels, at the nucleotide and at the chromosomal level (7). In GISTs DNA alterations play a major role in pathogenesis and disease progression (6,8-11). Different mutations in the *c-kit* or PDGFRA gene and other genes play a major role in tumor growth and resistance towards Gleevec® therapy (12,13). Chromosomal losses also play an important role, for instance allelic losses on chromosome 22 that occur independently from c-kit mutations (11).

Hox11L1, a gene involved in peripheral nervous system development, maps to human chromosome 2p (14). GISTs originate from the myenteric neuronal cells of *Cajal* (1). Congenital Cajal cell hyperplasia has been directly linked with neuronal intestinal dysplasia and *c-kit* gene mutation were excluded in these patients, suggesting other genes being involved (15). Defects in Hox11L1 gene have been linked with congenital intestinal neuronal dysplasia (16,17). Furthermore, Hox11L1 gene product is expressed by enteric neurons and mutated forms of Hox11L1 lead to myenteric neuronal hyperganglionosis and megacolon in mice (18-20).

Since Hox11L1 gene aberrations are clearly associated with Cajal cell hyperplasia, the aim of our study was to determine whether a relation of Hox11L1 with tumorigenesis in GISTs exists; in an attempt to find a new genetic marker with prognostic potential in GISTs, LOH on chromosome 2p near Hox11L1 was determined.

Materials and methods

Patients and samples. This study was approved by the Ethics Committee of the Chamber of Physicians in Hamburg, Germany. Written informed consent was obtained from all

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Table I. Clinicopathological characteristics of patients and Hox11L1 loss of heterozygosity (LOH) status in gastrointestinal stromal tumors (GISTs).

Variable	Hox11L1			
	No. of patients	LOH (%)	No LOH (%)	Non-informative (%)
GISTs	72	7 (10)	54 (75)	11 (15)
Sex				
Male	43 (60)	32 (74)	4 (9)	7 (16)
Female	29 (40)	22 (76)	3 (10)	4 (14)
Localization of primary tumor				
Esophagus	2 (3)	1 (50)	0	1 (50)
Stomach	50 (70)	40 (80)	4 (8)	6 (12)
Small intestine	16 (22)	10 (62)	3 (19)	3 (19)
Colorectal	3 (4)	2 (67)	0	1 (33)
Unknown	1 (1)	1 (100)	0	0

patients for use of the resected samples. For this study, 72 patients with GIST who were surgically treated between 1985 and 2004 were chosen retrospectively. In case of surgery before the year 2000, the diagnoses included various termi-nologies like schwannoma, sarcoma, leiomyoma, leiomyo-sarcoma, leiomyoblastoma and malignant peripheral nerve sheath tumor. Tumor samples from patients with mesenchymal tumors from the gastrointestinal tract that were possibly mis-diagnosed at times when diagnostic criteria for GIST were not established, were recently re-evaluated and re-classified retrospectively. Besides histological criteria, the following antibodies were used for immunohistological (re-)classification of all tumors: CD117 (*c-kit*; rabbit polyclonal) (Dako, Glostrup, Denmark), CD34 (mouse IgG1) (Novocastra Laboratories Ltd., Newcastle, UK), desmin (clone DE-R-11) (Dako), muscle actin (mouse IgG1; clone HHF35) (Enzo Diagnostics Inc., NY), and *S-100* protein (polyclonal) (Dako). The proliferative index was determined with *Ki-67* (*MIB1*; IgG1) (Dako) and was categorized as mitotic count of <5/50 high power fields (HPF), 5-10/50 HPF and >10/50 HPF. All data including sex, age, histopathological data such as size, lymph node metastases, tumor type and disease stage were obtained from the clinical and pathological records.

Detection of loss of heterozygosity at Hox11L1 gene locus. DNA was extracted from snap-frozen tumor or normal tissue samples at -70˚C using a standard extraction protocol (Qiagen, Germany). Before extraction, the tumor tissue samples were microdissected and judged by a pathologist to make sure that vital tumor tissue had been obtained. DNA (1 μg) was subjected to PCR using TaqGold polymerase. Primer sequences and locations were obtained from the Genome Database (NCBI). For allelic analysis of gene region near Hox11L1, primer D2S286 (GeneBank Accession: Z2350; TLX2 T-cell leukemia, homeobox 2; *homo sapiens* chromosome 2, maps: 2p13.1-p12; locus PARK3), was chosen with following primers: forward: 5'-TTAAAATTGTTTCTATGACATG ATG-3', reverse: 5'-TGGTGGTTTATCTTACCAGTC-3'. PCR product were 134-150 bp. Fluorescent dye-labeled primers were used (5'-labeled polynucleotides containing fluorescein/carboxyfluorescein, rhodamin/6-carboxytetra-methylrhodamin, HEX) (MWG, Ebersberg, Germany). Analysis was done using paired tumor and normal DNA. DNA was subjected to 40 cycles at a denaturing temperature of 94˚C for 30 sec, followed by 55˚C annealing temperatures for 30 sec and an extension step at 72˚C for 1 min using a thermocycler by Biometra (Goettingen, Germany). Amplification products were analyzed using the ABI PRISM Genetic Analyzer 310c (Applied Biosystems). Data were processed using GeneScan™ software. PCR was repeated at least 3 times in case of positive results and twice in case of negative testing. Normal DNA from the patient served as a negative control. When homozygosity occurred a patient was defined to be non-informative. LOH was defined as loss of allelic balance as described by PE Biosystems and previously reported (21). DNA from tumor samples and healthy tissue was also compared by gel electrophoresis.

Survival data. Clinical follow-up data were obtained by reviewing the hospital records, direct communication with the patient or attending physicians and from the Cancer Registry of Hamburg. All patients recruited into the study gave informed consent. Tumor-specific survival was calculated from the date of surgical excision of the tumor to the date of death or last follow-up. Patients who died from causes other than GIST were censored at the time of death. Patients whose death was clearly documented as attributable to GIST considered to have died of the disease; other deaths were not considered to have been caused by GIST. Recurrence-free survival was calculated from the date of surgery to the date of recurrence. Peritoneal carcinosis was found in 4 cases and distant

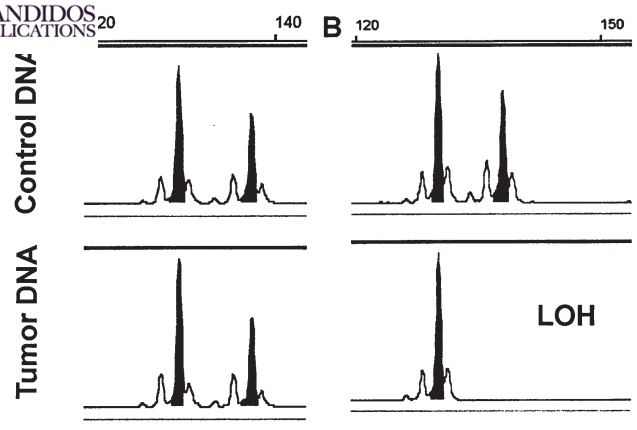

Figure 1. Normal and tumor DNA of two patients with GIST amplified with primer D2S286 located near Hox11L1 gene shows in one patient no allelic loss in tumor in comparison to normal control DNA (A), however, in another patient LOH (B; lower panel) was detected.

metastasis in 15 cases (liver metastasis in 14 patients and lung metastasis in 1 patient). Eleven (15%) of 72 patients were excluded for survival analysis as genomic analysis was non-informative. Of 2 patients no follow-up data could be generated, so that survival analysis finally included 59 patients.

Statistical analysis. SPSS for Windows (Version 11.5.1) (SPSS Inc., Chicago, IL) was used for statistical analysis. Survival curves were plotted using the Kaplan-Meier method and analyzed using the log-rank test. Significance statements refer to p-values of two-tailed tests that were <0.05. To assess the independent influence of presence of LOH simultaneously with covariates, Cox regression analysis for multivariate analysis was performed.

Results

Frequency of LOH in GIST patients. A total of 72 GIST patients were included in our study. Patient characteristics are listed in Table I. Briefly, the median age of GIST patients was 61 years. All 72 GISTs were *c-kit*-positive (100%), 64 (88.9%) were CD34-positive, 4 (5.6%) positive for *S-100* protein, 22 (30.6%) for smooth muscle actin and 3 (4.2%) for desmin. Fig. 1 shows representative analysis patterns for detection of allelic imbalance at Hox11L1 gene locus at chromosome 2. Of all 72 GIST patients included in this study, LOH was detected in 7 (10%) tumor samples. Non-informative status was encountered in 11 (15%) cases and meant that the normal tissue of the patient was displaying homozygosity, thus not allowing for LOH detection at that site. The remaining 54 (75%) did not show LOH.

Table II. Multivariate Cox regression analysis for tumor-specific survival for various factors.[a]

Variable	Relative risk (95% confidence interval)	P-value
Age	0.78 (0.22-2.75)	0.701
Sex	0.96 (0.91-1.01)	0.124
GIST classification	4.16 (1.04-16.61)	0.044[a]
LOH of Hox11L1	3.39 (0.98-11.76)	0.055

[a]GIST were classificied according to the 'consensus approach of 2002' that grades tumors according to tumor size and mitotic count.

LOH in GISTs and survival. Follow-up data of surgically treated GIST patients were generated. Median follow-up time of all patients included for survival analysis (n=59) was 37 months. The median follow-up time of survivors (n=39) was 43 months, of patients with LOH (n=7) was 43 months and of patients without LOH (n=52) 37 months (Fig. 2). Statistical analysis by log-rank test revealed that LOH adversely influenced tumor-specific and recurrence-free survival (p<0.05). Survival analysis of patients by Kaplan-Meier method revealed

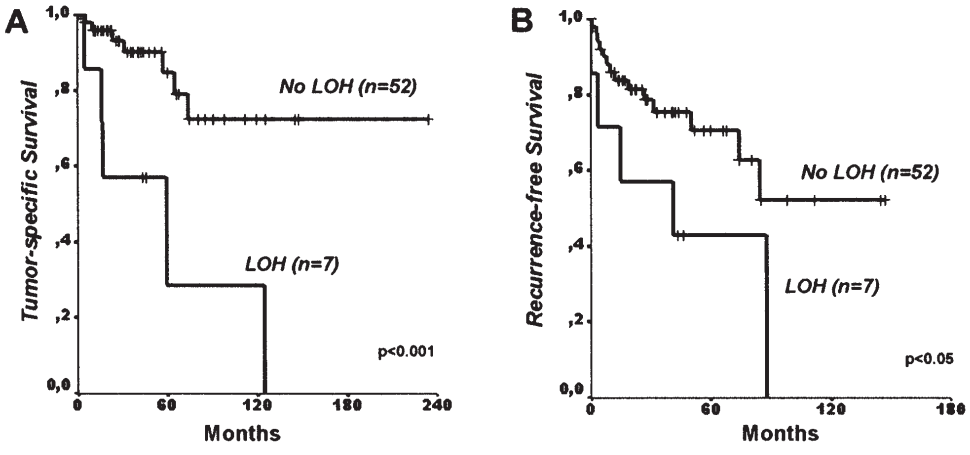

Figure 2. Kaplan-Meier curve for tumor-specific (A) and recurrence-free (B) survival and *LOH* at Hox11L1 gene locus on chromosome 2 in GISTs. Patients suffering from GIST that were surgically treated were included for survival analysis (n=59). P-values were calculated with log-rank test.

that a presence of LOH at Hox11L1 gene locus could predict a significant worsened tumor-specific and recurrence-free survival as assessed by univariate analysis ($p<0.05$ by log-rank test) and shown in Fig. 2. Among patients with LOH, 2 events of recurrence where noted, one developed liver metastasis and the other peritoneal carcinosis. Cox regression model for multivariate analysis was used to examine whether various factors were associated with reduced tumor-specific survival (Table II). The following covariates were included in the model as potential risk factors: age, sex, score recommended by the 'GIST consensus approach' of 2001 ('Fletcher' score) including tumor size and mitotic index, and LOH at Hox11L1 gene locus. Tumor-specific survival was independently associated with the 'consensus approach of 2002' that includes tumor size and mitotic rate [relative risk (RR)=4.16, 95% CI=1.04-16.61; p=0.044]. Presence of LOH at Hox11L1 gene locus had a relative risk of 3.39 for decreased tumor-specific survival, reaching borderline level of significance by multivariate analysis (RR=3.39, 95% CI=0.96-11.76; p=0.055). For age and sex no independent prognostic effect was found (Table II).

Discussion

The present study investigated allelic loss on chromosome 2 near Hox11L1 gene locus and its impact on survival in patients with GISTs that are originating from Cajal cells. Hox11L1 gene aberrations are associated with Cajal cell hyperplasia, and a role of Hox11L1 gene in tumorigenesis of GIST is proposed by this study.

The study shows the importance of LOH on chromosome 2. The impact of LOH on other locations, for instance chromosome 22 near NF2 gene, was described recently (9). LOH at the chromosome 2 reflects the course of the disease and alterations near Hox11L1 gene are indicative for impaired survival.

Hox11L1, a gene involved in peripheral nervous system development, maps to human chromosome 2p (14). GISTs originate from the myenteric neuronal cells of Cajal (1). Congenital Cajal cell hyperplasia has been directly linked with neuronal intestinal dysplasia and Hox11L1 gene absence in knock-out mice (15-17). Hox11L1 gene product is expressed by enteric neurons and mutated forms lead to myenteric neuronal hyperganglionosis and megacolon in mice (18-20). Our data show that Hox11L1 gene aberrations are associated with a malignant form of Cajal cell hyperproliferation, namely GISTs. Our results support that deletion near Hox11L1 is an early and presumably primary event in the tumorigenesis of GISTs. The observations of this study are in agreement with previous studies showing presence of LOH in different chromosomal regions in GISTs. Whether chromosome 2 deletion is specific for GISTs is unknown.

In an attempt to find a new genetic marker with prognostic potential in GISTs, this study showed that allelic loss of Hox11L1 gene region has also prognostic impact. p53 gene has a function in tumor growth and GISTs have been studied for protein expression of p53 (22). p53 was found to be differentially expressed in GISTs of different sites and to have an independently prognostic effect for gastric GISTs. Loss of p53 gene might also be causative for tumor progression in GISTs. Further cytogenetic studies need to follow.

Several classifications of GISTs exist, one of them is the 'consensus approach of 2002' that considers primary tumor size and mitotic rate (1). The outcome depends on these two parameters (6). However, even small tumors without mitoses can metastasize and other immunohistochemical markers, e.g. proliferation marker Ki-67, still fails to be independent prognostic markers. Although *c-kit* expression is the established standard in the identification of GISTs, additional prognostic and differentiation markers to other tumors of mesenchymal origin are needed. Studies using DNA microarray technique have revealed a distinct gene expression profile in GISTs allowing distinguishing these tumors from mesenchymal malignancies (23-25). A specificity of Hox11L1 gene loss for GISTs has yet to be determined.

Also, under the assumption, that tumors shed DNA into the blood stream, a therapeutic monitoring of GIST is conceivable. A similar approach in squamous cell cancer of the esophagus has been performed (21). For instance for GIST patients receiving Imatinib, DNA alterations detected in the peripheral blood could aid to detect response to therapy.

In summary, our results indicate that presence of LOH on chromosome 2 at Hox11L1 gene locus has a negative impact on survival and is associated with malignant phenotype GIST. A role of Hox11L1, a gene known to be associated with hyperplasia of intestinal neuronal cells, in tumorigenesis of GISTs can be postulated. This has to be explored in further molecular genetic studies.

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