Molecular Cytogenetics of Bone and Soft Tissue Tumors


INTRODUCTION

Many different neoplasms may arise from the various cell types that are present in human supportive tissues. The term "soft tissue tumor" is used to denote a subgroup of these neoplasms and includes synovial sarcomas, fibrosarcomas, liposarcomas, leiomyosarcomas, rhabdomyosarcomas, angiosarcomas, and the most common form, malignant fibrous histiocytomas [1]. Cytogenetic studies have revealed the occurrence of both numerical and structural abnormalities in several of these tumors, including recurrent chromosomal translocations [2-5]. In other soft tissue tumors, double minutes, ring chromosomes, and large rod-shaped markers have repeatedly been encountered. These latter anomalies appear to be associated with gene amplifications [6-8]. Also, osteosarcomas originate from supportive tissues [9]. At present, little is known about the cytogenetic alterations that occur in these tumors or how such alterations may relate to tumor initiation and/or progression [2]. However, some studies have revealed highly aneuploid and extremely complex karyotypes with numerous cytogenetic abnormalities including, again, supernumerary ring chromosomes and double minutes [10-12].

Here, on the occasion of Dr. Avery Sandberg's 75th birthday, the recent molecular genetic analysis of a soft tissue tumor (synovial sarcoma)-associated chromosomal translocation and the delineation via comparative genomic hybridization of more complex cytogenetic anomalies in several other (sub) types of human bone and soft tissue tumors are discussed.

MOLECULAR GENETIC ANALYSIS OF t(X;18)(p11.2;q11.2) IN HUMAN SYNOVIAL SARCOMAS

Synovial sarcoma is a soft tissue tumor that predominantly afflicts adolescents and young adults [1]. Despite their designation, it is now generally accepted that these tumors may not originate from synovial tissue but, instead, from multipotent stem cells that are capable of differentiating into mesenchymal and/or epithelial structures [1, 13]. Furthermore, in spite of its predominant occurrence in the lower and upper extremities, synovial sarcomas may also be found in several other body locations such as head, neck, and trunk. Based on the histopathological appearance, synovial sarcomas can be divided into biphasic, monophasic fibrous, or monophasic epithelial (very rare), and poorly differentiated tumors [1]. Among several numerical and structural cytogenetic abnormalities, also a recurrent translocation between the chromosomes X and 18 (Figure 1) has been documented [14]. A compilation of all cytogenetic studies performed to date indicates that this t(X;18)(p11.2; q11.2) is present in more than 90% of the synovial sarcomas, in about one third of the cases as the sole cytogenetic anomaly [2, 4]. The vast majority of primary tumors exhibit a near diploid karyotype and, compared to locally recurring and metastatic lesions, are less likely to carry additional secondary aberrations [15].

Considering a putative causal relationship between the occurrence of t(X;18)(p11.2;q11.2) and synovial sarcoma development, studies were carried out to characterize the chromosomal breakpoint in detail and to isolate the genes involved. Based on fluorescence in situ hybridization (FISH) analysis with X chromosome-specific yeast artificial chromosome (YAC) probes, we and others identified two alternative Xp11.2 breakpoints, one within the ornithine aminotransferase-like 1 (OATL1) pseudogene cluster and another one close to the related OATL2 cluster [16, 17]. Accordingly, a subgroup of tumors showed a split fluorescent signal when hybridized with an OATL1-specific YAC, but not with an OATL2-specific YAC. The opposite was observed in the other subgroup of tumors, indicating that the two alternative Xp11.2 breakpoints are mutually exclusive [18]. The subsequent subcloning of one of the YACs (OATL1) resulted in probes that hybridize to altered...
restriction fragments in tumor DNAs after Southern blot analysis. These probes, in turn, were used to isolate a chimeric breakpoint fragment containing both chromosome X and chromosome 18-derived sequences [19, 20]. Further positional cloning studies led to the isolation of the genes that are disrupted in t(X;18)(p11.2;q11.2)-positive synovial sarcomas. It was found that, in conformity with the previous findings, one of these genes (SYT; chromosome 18) becomes fused to one of two alternative X chromosomal genes (SSX1 or SSX2) located in the OATL1 and OATL2 regions, respectively [21-23].

The normal SYT gene transcript (mRNA) measures about 4.0 kb (Figure 2) and encodes a 387 amino acid peptide rich in glutamine, proline, and glycine. The deduced protein shows no overt homologies to known protein sequences, with the exception of three potential SH2 binding domains and one potential SH3 binding domain [21]. These domains have been implicated in protein-protein interactions related to signal transduction [24]. Recently, the mouse homolog of SYT was cloned and, through database searches, annexin-like sequence repeats (also present in human SYT) were found which, again, may be involved in protein-protein interactions [25]. SYT is an ubiquitously expressed gene. In the adult mouse, SYT mRNAs are present in several organs, most markedly in heart, kidney and testis. RNA in situ hybridization experiments have shown that during mouse embryonic development, SYT expression turns from ubiquitous at initial stages into more restricted to cartilaginous and specific neuronal cells and some epithelial derived tissues, at later stages [25]. The SSX1 and SSX2 gene transcripts are approximately 1.6 kb long and encode highly homologous proteins (81% identity over 188 amino acids) [23]. These proteins are rich in charged amino acids, including an acidic C-terminal tail, and contain consensus sequences for N-glycosylation and tyrosine phosphorylation. Notably, the SSX protein sequences contain N-terminal domains which exhibit extended homologies to the so-called Krüppel-associated box (KRAB) originally found in the Drosophila Krüppel gene product. KRAB domains have also been found at the N-terminal ends of a subgroup of zinc finger proteins and are thought to be involved in transcriptional repression [26]. Data base searches revealed that SSX2 is identical to a gene (designated HOM-MEL40) that was independently isolated by others, based on its abundant expression in melanomas [27]. Normally, the SSX genes exhibit very restricted expression patterns, with transcripts mainly in human testis and thyroid [23]. The SYT-SSX fusion RNAs are about 2.4 kb long (Figure 2) and, in the majority of cases, encode proteins in which the last eight amino acids of SYT are substituted for the last 78 amino acids of SSX. Some rare variants have also been described which carry more N-terminally located breakpoints either in SYT or SSX [22, 23]. It is noteworthy that all the reported fusion transcripts exclude the putative KRAB domain present in SSX and at least one of the putative SH2 binding domains present in SYT. It is assumed that these translocation-associated ablations have functional implications for the development of synovial sarcomas.

The overall uniformity demonstrated in chimeric transcripts indicates that the chromosomal breakpoints must be clustered within specific introns in the SYT and SSX genes. We have mapped six breakpoints within the SYT gene and found that indeed all of them were located within the same intron just upstream to the exon encoding the last eight amino acids (Figure 3). Very similar results were obtained by others [23]. Analogous attempts to map breakpoints on the X-chromosome were hampered by the fact that this region harbors various (low-copy) repetitive elements. As a corollary of our ongoing work, we recently cloned a novel SSX gene, designated SSX3, also located on the X-chromosome but apparently not implicated in t(X;18)(p11.2;q11.2)-positive synovial sarcomas. In addition, we found indications for the existence of at least two more SSX-related genes, SSX4 and SSX5, within the same region [28]. Also, in another study, evidence has been provided

Figure 1 Partial synovial sarcoma-derived karyotype showing the normal chromosomes X and 18 and its translocation derivatives X/18 and 18/X.

Figure 2 Northern blot analysis of three synovial sarcomas (lanes 1–3) and two normal control tissues (lanes 4 and 5) using a SYT cDNA as probe. The positions of the SYT and SYT-SSX mRNAs are marked.
for the presence of multiple SSX genes in Xp11.2 [29]. These observations are in agreement with the repetitive nature of this genomic segment.

Some characteristics of the (t(X;18)(p11.2;q11.2) and the SYT-SSX fusions are comparable with those pertaining to other sarcomas, i.e., a widely expressed gene on one chromosome becomes juxtaposed to a transcription factor gene with a restricted expression pattern on another chromosome, resulting in a fusion transcript encoding an activator of transcription [30]. Even though SSX sequences become expressed when fused to SYT and share some features typical of transcription factors, a definite role for the SYT-SSX fusion proteins in transcriptional control still has to be ascertained.

Considering that (t(X;18)(p11.2;q11.2) is specifically present in synovial sarcomas, its value as a diagnostic marker seems to be unquestionable. The use of YAC probes for FISH analysis on fresh or archival tumor samples has amply proven its usefulness in the detection of the translocation and the localization of the Xp11.2 breakpoints, even when complex rearrangements are present [18, 31]. In addition, the isolation and characterization of the SYT and SSX genes has opened up the possibility of employing a reverse transcription polymerase chain reaction (RT-PCR) assay as a means to detect SYT-SSX fusion transcripts and, therefore, to retrieve information from tumors not amenable to cytogenetic analysis. By using the RT-PCR approach, SYT-SSX transcripts have been detected in 70 synovial sarcomas—45 with SYT-SSX1 transcripts and 25 with SYT-SSX2 transcripts [22, 23, 32, 33]. Both techniques, FISH and RT-PCR, have also been used to evaluate a possible correlation between Xp11.2 breakpoint localization and histological tumor (sub)type. Interestingly we found that all biphasic synovial sarcomas exhibited a break in the OATL1 (SSX1) region, whereas most tumors diagnosed as monophasic were found to carry a break in the OATL2 (SSX2) region [18, 34]. These latter findings have been corroborated by some but not all reports from other investigators and, as such, must await further validation [32, 33, 35].

**Figure 3** Partial genomic map of the SYT gene and the positions of six synovial sarcoma-associated breakpoints (arrows). The last SYT exon is marked by 3' end. X = XbaI; B = BamHI; H = HindIII; E = EcoRI.

**Figure 4** Partial metaphase spread of a soft tissue tumor (malignant fibrous histiocytoma) showing supernumerary ring chromosomes (arrows).
substantial number of cases [39–41]. Since MDM2 appears to inactivate the tumor suppressor TP53, a growth advantage via 12q13–q14 amplification has been attributed to increased copy numbers and expression levels of the MDM2 gene [39, 42]. In osteosarcomas, MDM2 amplifications have been detected specifically in recurrent and metastatic lesions, and it has been suggested that such amplifications may be associated with a poor prognosis [43]. Other genomic alterations include loss or rearrangement of the chromosome 13q14 and 17p13 subregions harboring the RBI and TP53 tumor suppressor genes, respectively [44–48]. It has been shown that alterations at the RBI locus in primary osteosarcomas may serve as indicators for a poor clinical outcome [49].

In order to get further insight into the molecular cytogenetic changes that are relevant for tumor growth, the recently developed comparative genomic hybridization (CGH) technique [50–52] was employed to map regions of amplification in a large group of bone and soft tissue tumors of various histologic (sub) types. The CGH technique provides information not only on the overall occurrence of amplifications, but also on the extent of the regions affected [51, 53–55]. The results obtained are instrumental in the ultimate identification and isolation of the genes involved.

Through CGH, DNA amplifications were detected in different soft tissue tumors, including fibrosarcomas, liposarcomas, leiomyosarcomas, and malignant fibrous histiocytomas [52, 56, unpublished results]. The sizes of the amplified regions varied considerably, as did the number of amplified regions detected in each individual tumor sample. In total, 14/60 soft tissue tumors showed amplifications at 12q14. In some cases the amplifications included, besides 12q14, parts of 12q13 or 12q15. Also, other regions of chromosome 12 were amplified, although less frequently [52]. Amplicons covering 12q21-q22 were detected in four cases and amplification of the 12q24 segment was detected in two cases. A specific region on the long arm of chromosome 1 also showed frequent increases in sequence copy numbers. Although the different amplicons coinciding within this region were variable in size, they showed a minimal overlap at 1q21-q22. Amplification of this segment was observed at least as frequently as the 12q14 amplification. In addition, the occurrence of the 1q21-q22 and 12q14 amplicons appeared to be unevenly distributed among the different soft tissue tumor subtypes. However, no clear correlation between the presence of either 1q21-q22, 12q14, or both amplicons and any of the currently known clinical parameters has been observed. Therefore, it remains to be established what the biological consequences of the various combinations may be. In total, amplifications involving 25 different chromosomal (sub) regions were observed in the soft tissue tumors studied.

In 5/20 osteosarcomas, CGH revealed gain of whole chromosomes or chromosome arms, and in three of these cases, 6p was involved. Regional copy number increases were repeatedly seen at 1q, 8q and 17p [57, unpublished results]. Again, the 1q amplicons were variable in size, but showed a minimal region of overlap at 1q21-q23 (three cases). Overrepresentation of 8q23-pter, a region in which the MYC gene maps, was observed in 4 cases, including one that showed gain of the whole 8q arm. Amplifications of the 17p11–p12 region (Figure 5) were detected in five cases, including one involving the whole 17p arm. Overall, CGH analysis revealed high-level DNA amplifications at 23 different chromosomal sites in primary as well as metastatic osteosarcomas.

The majority of the bone and soft tissue tumors mentioned above were also assayed by Southern blot analysis for amplifications of the 12q14-linked proto-oncogenes MDM2, CDK4, SAS, CHOP, GLI and A2MR [39, 58, 59]. CGH analyses revealed increased DNA-sequence copy numbers in all the cases with known gene amplifications, as expected. Also, in a few cases without known gene amplifications, 12q14 amplicons were detected. A role as a selective driver in these amplicons was previously assigned to the MDM2 or CDK4 genes [39, 40, 58, 60]. However, the CGH results suggest that also alternative explanations should be considered, including amplification via a mechanism driven by another gene(s) located in band 12q14 [61, 62].

CGH analysis revealed two novel major regions of amplification, namely 1q21-q22 and 17p11-12. Increased sequence copy numbers coinciding with the 1q21-q22 segment were observed in 14 soft tissue tumors and four osteosarcomas. So far, this amplification was not observed in other malignancies such as prostate cancer or malignant

Figure 5 Comparative genomic hybridization analysis (upper panel) and fluorescence intensity profiles (lower panel) of an osteosarcoma exhibiting a 17p11-p12 amplicon. Tumor DNA was labeled in red and normal DNA in green. The chromosomes were counterstained using DAPI. The CGH ratio profile is marked in blue.
glioma [53, 63], nor has it previously been associated with the development of human sarcomas. Conceivably, the 1q21-q22 region may harbor a gene(s) important for human bone and soft tissue tumor initiation and/or progression. Several candidate genes have been mapped to this region, including the octamer-binding transcription factor OTF1 (1q22-q25) and various members of the S100 family of calcium-binding proteins, e.g., CACY and CAPL (1q21-q25) [64, 65]. Overexpression of the CACY gene has been associated with metastatic behavior of human melanomas [66]. High level amplifications at 17p11-p12 were detected in five out of 20 osteosarcomas. Very similar results were recently reported by Tarkkanen et al. [67]. This same region was found to be affected only sporadically in malignant astrocytomas [68; Hulsebos, personal communication] and in one of the soft tissue tumors that we tested [52, 56], rendering this anomaly into a highly specific one for osteosarcomas. So far, the 17p11-p12 segment does not seem to harbor any obvious candidate gene(s). Future positional cloning studies should be aimed at the identification of the genes related to cancer development within these regions.

Although the CGH technique is very well suited for the detection and mapping of DNA-sequence copy number changes, especially amplifications, one should be aware of its limitations. For example, CGH may fail to detect low-level copy number changes. Also, the total length of amplified DNA has to be of a certain size in order to be detectable [51, and references therein]. Thus, the actual amplification frequencies may be significantly higher than provided here, especially for the smaller amplicons. A more complete overview of genetic alterations in a given series of tumors can be obtained by combining CGH with FISH and other molecular genetic techniques using probes that specifically target the genomic regions highlighted by CGH.

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