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Clonality studies in sacral chordoma

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Abstract

Chordomas are rare, slow-growing, primary malignant skeletal neoplasms. Chromosome analysis, telomere reduction and telomere activity, DNA microsatellite, and loss of heterozygosity studies have been performed on chordomas; however, the clonality status (monoclonal versus polyclonal proliferation) is unknown. The primary purpose of this study was to determine whether sacral chordoma is monoclonal or polyclonal in origin with the use of a polymorphic X-linked gene (*AR*; alias *HUMARA*) and X-chromosome inactivation studies. DNA was harvested from tumor and corresponding normal tissue from eight women (37–71 years) with chordoma. Clonality was determined using an X chromosome inactivation protocol and a polymorphic human androgen receptor gene (*AR*) located on the X chromosome. The procedure required a methylation-specific polymerase chain reaction (PCR) and determination of the ratio of active to inactive X chromosomes. Results were informative for seven of the eight women, with two separate X-linked alleles seen for the *AR* gene in the normal tissue. Expression of *AR* gene alleles from each of the two X chromosomes was present in the chordoma tumor, indicating a polyclonal proliferation in all seven women. Most solid tumors and skeletal neoplasms are polyclonal in nature. Our study indicates that chordoma is polyclonal in its pattern of proliferation.

1. Introduction

Chordomas are rare, slow-growing primary malignant skeletal neoplasms that arise from remnant notochordal tissue. Reaching maturity in the embryo at 11 mm, the notochord obliterates and is displaced from the central to the cranial and caudal positions. Microscopic foci remain in the vertebral bodies at the cranial and caudal ends of the embryo. Malignant transformation typically occurs in the third to fourth decades of life for sphenoccipital lesions and in the fifth to sixth decades for the sacrococcygeal type. Overall, chordomas represent <1% of skeletal neoplasms, with an annual U.S. incidence of ~25 afflicted persons [1].

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Genetic studies performed on chordomas include chromosome analysis, telomere reduction and telomere activity, DNA microsatellite, and loss of heterozygosity (LOH) studies [2–6]. Various cytogenetic and molecular findings indicate 1p36 loss as a consistent change in sporadic and inherited chordomas [2]. In addition, microsatellite instability (MIN) and LOH studies performed on 12 chordomas detected MIN in 50% of patients at one or more loci, and LOH was identified in two chordomas, one of which had corresponding MIN [3].

Numerical and structural alterations in chromosomes 3 and 21 have also been observed. Many cases showed a hypodiploid or near diploid chromosome number [4]. Sandberg and Bridge [7] presented an exhaustive update on the cytogenetic and molecular alterations of chondroid neoplasms; they noted that about half of all chordomas show chromosome aberrations of diverse nature, suggesting that these alterations occur as late events in tumor progression. The retinoblastoma (*RB*) gene is a well-characterized tumor-suppressor gene whose protein binds nuclear DNA and plays a key role in cell-cycle regulation. Inactivation of the *RB* gene has been associated with a number of malignant neoplasms. Chordomas have demonstrated LOH at intron 17 of the *RB* gene in two of seven samples studied [5].

Analysis of the chromosome telomeres from chordomas have revealed lengthening in four of four samples [6]. In marked contrast, telomere length reduction has been observed during in vitro senescence of human fibroblasts and most cancers [8]. Telomerase, the enzyme responsible for maintenance of telomere length, was identified in about one-half of chordomas studied to date [6].

In general, tumors derived from the hematopoietic system (e.g., lymphoma) have a monoclonal genetic pattern. In contrast, the highly complex karyotypes of human solid tumors, which are often caused by imbalanced chromosomal alterations, make molecular targeted approaches to disease classification and treatment far more difficult than in hematopoietic neoplasia, for which many tumors have balanced chromosomal rearrangements [9]. It not known whether sacral chordomas, a primary skeletal neoplasm, represent monoclonal or polyclonal proliferation.

The human androgen receptor gene (*AR*; alias *HU-MARA*) is located on the X chromosome. This gene, which contains a highly polymorphic CAG trinucleotide repeat, can be used to assess clonality by determining the number of alleles representing the X chromosome, along with the inactivation status. In females, one of the two X chromosomes becomes inactivated (methylated) early in embryo development, and each subsequent cell has the same X chromosome active or inactive, as proposed in the Lyon hypothesis [10]. Therefore, if the X chromosomes are polymorphic for an X-linked gene, one can determine whether a collection of tumor cells contains a mixture of different active (unmethylated) and inactive (methylated) X chromosomes, thus establishing the clonality status (i.e., polyclonal versus monoclonal cell proliferation). If the same X chromosome is consistently active (or inactive), then only one X chromosome allele would be detected with molecular genetic techniques and the tumor cells would be judged to have a monoclonal proliferation (i.e., one DNA fragment band present, compared with two polymorphic DNA bands seen in the normal cells from the same patient).

Hence, to further understand the tumorigenesis of chordomas, we conducted a clonality study using a novel methylation-specific PCR analysis of the polymorphic X-linked *AR* gene. We conducted X inactivation studies to determine the polyclonal versus monoclonal nature of the tumor in eight women available for study.

2. Methods

2.1. Sample collection and DNA isolation

Because of the X chromosome inactivation studies, only females with sacral chordoma were included. After confirmation of the pathological diagnosis, archival paraffin-embedded tumor blocks were available from eight women (37–71 years). Genomic DNA was isolated from paraffin-embedded tumor sections using an EX-WAXDNA extraction kit according to the manufacturer's directions (Intergen, Purchase, NY). To avoid tissue contamination, tumor tissue was clearly identified in cross section on the paraffin-embedded sections before dissection was begun. The tumor cells were then carefully dissected by scraping the central tumor mass (not the periphery) with a fine point scalpel and placed on the microscope slides. In addition, control DNA was obtained from tumor-free tissue sections from the same tissue blocks in a similar fashion.

The study was approved and all procedures were performed in accordance with the ethical standards of the Springfield Illinois Committee on Research in Human Subjects and the Helsinki Declaration of 1975, as revised in 1983.

2.2. Methylation-specific PCR

A polymerase chain reaction (PCR)-based assay on methylation of the polymorphic X-linked human androgen receptor gene *AR* to assess clonality was used with established protocols [11,12]. Control and tumor DNA (0.2–1 µg) from each patient was treated with sodium bisulfite to convert the unmethylated allele from the active X chromosome; no conversion occurs on the methylated allele from the inactive X chromosome. In this treatment, cytosines are changed to uracils using the CpGenomeDNA modification kit (Intergen). Modified DNA was resuspended in 25 µL of TE (10 mmol/L Tris—0.1 mmol/L EDTA, pH 7.5).

PCR was performed with previously published primers specific for methylated (*M*) versus unmethylated (*U*) DNA [11]. The *M* primer sequence was designed for exon 1 of the *AR* gene against the unmodified DNA sequence of the methylated inactive X chromosome copy. The *U* primer set was designed for amplification of the modified unmethylated alleles on the active X chromosome. One primer from each primer set was end-labeled with ³²P to allow quantization. The polymerase was activated at 95 °C for 10 min. The DNA was amplified in a Perkin-Elmer (Fre-mont, CA) model 9600 thermocycler for 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The PCR products were mixed with loading dye and then separated on a 12% polyacrylamide gel. The gel was dried, exposed to a phosphor screen, and scanned using the Cyclone phosphor storage system.

Band intensities were determined and recorded using Perkin-Elmer Optiquant analysis software. If two distinct polymorphic bands were present, representing two different X chromosomes for the *AR* gene in the normal tissue, then clonality studies were performed on the tumor cells from the same female. If only a single X chromosome band was seen in normal cells, the subject was considered uninformative and no additional testing was done. The *AR* gene is > 80% informative: that is, the maternal and paternal X chromosomes can be distinguished in 80% of women [11].

3. Results

One of our eight women was uninformative due to homozygosity at the *AR* locus—that is, only one DNA band was seen in the normal tissue. This woman was 55 years old. The eight women with chordoma ranged in age at the time of diagnosis from 37 to 71 years (mean age: 52). In the remaining seven patients, polymorphisms at the *AR* locus were present in the normal tissue, as demonstrated by the presence of two different DNA bands representing each X chromosome (Fig. 1). As expected, there was some variation in X chromosome inactivation ratio and the intensity of the two DNA bands representing each X chromosome in the normal tissue (i.e., not every tissue sample had a 50:50 ratio of inactivation for the X chromosomes). Similar analyses of the tumor DNA in these women revealed dual banding patterns for all cases, indicating the presence of expression of each X chromosome with a mixture of active and inactive X chromosomes. In each of the seven informative cases, two bands were present and the ratio of the two DNA signals ranged between 1:1 (normal and tumor tissue for Patient 3) to 2.7:1 (normal tissue for patient 7) (Fig. 1). These results involved a mixture of expression representing each of the two X chromosomes in the chordoma cells, thus derived from more than one clone of cells and polyclonal in nature (Fig. 1).

4. Discussion

The Lyonization model states that, in females, either the paternal or maternal X chromosome is inactivated with equal probability during mitosis when a cell divides to produce its progeny [10]. Although random inactivation occurs in the majority of females, preferential selection occasionally results. Female carriers of X-linked diseases can demonstrate nonrandom or skewed X chromosome inactivation patterns and have symptoms of the disease (often referred to as poor Lyonization). In these cases, the nonmutated X chromosome is thought to inactivate disproportionately in the target cells, which allows for over expression of the mutant X-linked gene. As demonstrated in our study, the X chromosome inactivation pattern can be used to study clonality in neoplasms with the use of X-linked genes that are polymorphic (such as the *AR* gene) and rapid PCR methylation sensitive assays. Seven of the eight women studied with sacral chordomas showed the presence of two different alleles of the *AR* gene in the normal cells, thus permitting clonality studies to be performed in the neoplastic cells. In all seven informative female patients, two different bands were observed, representing the expression of each of the two different X chromosomes in the tumor cells indicating a polyclonal nature of chordoma tumor development.

Knowing the clonality status of tumors may affect diagnosis and potential treatment of tumors. For example, monoclonality offers a pharmacologic therapeutic opportunity, because the cells are likely to behave identically. Unfortunately, polyclonal tumors offer greater challenges, in that multiple strategies are needed to obtain cytotoxicity.

Sacral chordoma grows slowly, with vague and nonspecific symptoms appearing late in the disease course. Metastasis is a late finding in 10% of cases. The only current therapeutic option is sacral amputation with wide surgical margins. Nerve root compromise is common with or without surgery, necessitating bowel and bladder diversion.

This study indicates that chordoma cells proliferate polyclonally, confirming that the physaliferous tumor cells are of mesenchymal rather than hematopoietic origin. Although previous studies have indicated disturbed patterns of DNA replication and the peculiar presence of abnormally long telomeres [6], a well-defined molecular basis for this cancer is lacking. More studies are needed to understand the molecular biology of this rare and sometimes fatal cancer.

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Fig. 1.

Clonality analysis of chordomas from women determined to have two AR (alias HUMARA) alleles in normal tissue and dual DNA bands in the tumor tissue, representing X chromosome inactivation from the same subjects. The presence of two DNA bands from each of the two separate active X chromosomes in the tumor cells indicates polyclonal cell proliferation. DNA band intensities were quantified using Optiquant analysis software and reported as digital light units (DLU). N, normal tissue; T, tumor tissue.