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Original contribution

Somatic mutations of the mitochondrial genome in Chinese patients with Ewing sarcoma☆,☆☆

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Summary Somatic mutations in mitochondrial DNA (mtDNA) have been long proposed to drive initiation and progression of human malignancies. Our previous study revealed a high prevalence of somatic mutations in the D-loop region of mtDNA in Ewing sarcoma (EWS). However, it is unclear whether somatic mutations also occur in the coding regions of mtDNA in EWS. To test this possibility, in the present study, we sequenced the whole mitochondrial genome from 20 cases of EWS specimens and their corresponding peripheral blood samples. We identified a total of 6 somatic mutations in the mtDNA coding regions in our EWS series, and 5 of them were missense or frame-shift mutations that have the potential to directly influence proper mitochondrial function. In combination with our earlier observations on the D-loop fragment, 70% (14/20) of EWS tissues appeared to harbor somatic mtDNA mutations. Among the identified 25 somatic mutations, 19 (76%) were located in the D-loop control region, 1 (4%) was in the sequence of the $tRNA^{Val}$ gene, 1 (4%) was in the mitochondrial ATP synthase subunit 6 gene, and 4 (16%) occurred in genes encoding components of the mitochondrial respiratory complexes. In addition, patients carrying somatic mtDNA mutations did not show significant association with their clinicopathologic characteristics. Together, these findings suggest that somatic mtDNA mutations occur in both protein coding and noncoding regions of mtDNA, which may play critical roles in the pathogenesis of EWS and should be further explored for its possible use as a novel marker for monitoring EWS occurrence and advancement.

1. Introduction

Ewing sarcoma (EWS) is the second most common bone-associated malignancy, primarily afflicting children and young adults in the first 2 decades of life [1]. Ewing sarcoma histopathologically comprises sheets of undifferentiated small, round, blue cells with a high nuclear-to-cytoplasmic ratio [2]. Genetically, EWS is hallmarked by nonrandom aberrant

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chromosomal rearrangements involving the *EWS* gene on 22q12 and fusion partners from the ETS (E-twenty six) family of transcription factors, most frequently *FLI1* (Friend Leukemia Integration 1) on 11q24 (~85%) [3]. This highly aggressive tumor has a marked propensity for systemic spread, and ~25% of affected individuals present with detectable metastasis at diagnosis [1]. Despite major advancements in early detection and multimodal regimens combining local surgery, radiotherapy, and intensified multiagent chemotherapy, the overall survival for those with metastatic or recurrent disease has hitherto remained stagnant at a very dismal 10% to 20% [4]. This fact underscores an urgent need to develop novel and more effective strategies for early diagnosis and better treatment of this fatal disease.

Mitochondria are semiautonomous organelles in eukaryotic cells and play pivotal roles in energy metabolism, free radical production, and apoptosis [5]. Human mitochondrial DNA (mtDNA) is a 16 569-base pair (bp), closed-circular, duplex DNA molecule that encodes 13 polypeptide subunits of the oxidative phosphorylation (OXPHOS) system, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) essential for intramitochondrial protein synthesis [6]. Compared with nuclear DNA (nDNA), mtDNA is present at extremely high levels in each individual cell with up to 10^3 to 10^4 copies [7]. Mitochondrial DNA contains a unique 1124-bp noncoding segment called displacement (D)-loop with regulatory sequences responsible for maintaining mtDNA transcription and replication [6]. Owing to limited DNA repair capacity, the lack of histone protein protection, and its constant exposure to high levels of endogenous reactive oxygen species (ROS) in the mitochondrial inner membrane, mtDNA is notably vulnerable to oxidative or other genotoxic damages and thus has a much higher mutation rate (10- to 200-fold) than nDNA [8]. In addition to a heavy load of common sequence changes in nDNA, somatic mtDNA mutations have been increasingly discovered in various primary human cancers, with the highest frequency in the D-loop control region, and have been proposed as a promising molecular biomarker for monitoring tumor initiation and progression [9]. Accumulation of mtDNA mutations may potentially compromise proper function of the OXPHOS system and induce generation of additional ROS, which, in turn, accelerates the rate of mtDNA mutations [10]. It has been widely accepted that this vicious cycle contributes to the etiology of human cancers [10].

In our earlier studies, we have demonstrated that EWS tumor specimens harbored a high frequency of somatic mutations in the D-loop region of mtDNA, preferentially in the 2 hypervariable segments (HVS1 and HVS2) as well as the homopolymeric C stretch between nucleotide positions 303 and 309, and carried lower mtDNA copy number as compared with normal bone tissues [11,12]. Furthermore, somatic D-loop mutation is likely a crucial factor, giving rise to mtDNA quantitative changes in EWS [12]. To further scrutinize the prevalence and distribution of mtDNA mutations in EWS, in this study, we sought to examine somatic mutations along the complete mitochondrial genome sequence in 20 cases of patients with EWS and speculated on the functional consequences of these mutations.

2. Materials and methods

2.1. Study subjects and tumor samples

Twenty patients (13 male, and 7 female) who were diagnosed as having EWS and received surgical resections at the Department of Bone and Soft Tissue Tumor, Tianjin Medical University Cancer Hospital, and at the Department of Surgical Oncology, Central Hospital of China National Petroleum Corporation, between 1980 and 2009 were enrolled in this study. Seventeen of the 20 patients are the cohort reported previously [11], and D-loop sequence alterations in these individuals have been screened by adopting high-fidelity polymerase chain reaction (PCR) and direct sequencing. The median age of patients was 15 years (range, 1-25 years), and none of them received preoperative treatment. Primary tumors were located in the pelvis (n = 7), the femur (n = 5), the fibula (n = 2), the tibia (n = 2), the humerus (n = 1), the rib (n = 1), the spine (n = 1), and the foot (n = 1). Six people had metastasis at initial diagnosis. All tumor specimens were histologically evaluated by 2 independent experienced pathologists. The size of tumor was measured as the maximum tumor diameter on radiographic images including computed tomography scans and magnetic resonance imaging. Tumor tissues were snap frozen in liquid nitrogen immediately after surgical resection and stored at −80°C until further use. As references, 5 to 10 mL of peripheral blood samples were drawn from all subjects and kept at −80°C before DNA extraction. All samples in this study were obtained with informed consent, and this study was approved by the Review Board of the Hospital Ethics Committee.

2.2. DNA extraction

Total DNA from EWS tumor tissues was prepared by digestion with 0.2 mg/mL proteinase K and 1% sodium dodecyl sulfate, followed by phenol/chloroform extraction and ethanol precipitation using standard protocols. Total DNA from 2 mL of blood samples was extracted by using a QIAamp DNA blood Midi Kit (Qiagen, Shanghai, China). The final DNA was dissolved in sterilized Milli-Q water and quantified on a NanoDrop spectrophotometer (Fisher, Wilmington, DE, USA).

2.3. PCR amplification of the entire mitochondrial genome

Total DNA was subjected to PCR amplification using 11 pairs of primers previously designed by Levin et al [13]. These primer sets generated overlapping DNA fragments between 982 and 2134 bp that span the full-length sequence