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Original Article

Mutational landscape implicates epithelial-mesenchymal transition gene TGF-β2 mutations for uterine carcinosarcoma after adjuvant tamoxifen therapy for breast carcinoma

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Abstract: Uterine carcinosarcoma (UCS) is a rare aggressive malignancy. Several reports previously described UCS occurring after tamoxifen therapy for breast carcinoma. However, the genetic landscape of tamoxifen-related UCS remains unclear. We performed whole-exome sequencing of two UCSs after tamoxifen therapy for breast carcinoma to determine mutational profile of UCSs and those corresponding breast carcinomas. Our results demonstrated that 374 somatic variants in 141 genes were shared across the two UCSs, whereas no shared somatic variations across the breast carcinomas were found. Pathway analysis indicated the MAPK pathway, including the epithelial-mesenchymal transition (EMT) inducer gene TGF-β2 mutations (c. 1039G > A and c. 1040C > T, both p.A347T), recurrently occurred in UCS, while ER-related gene EP300 (p.P16L) and ESR1 (p.V355I) mutations were identified independently in breast carcinomas. These findings highlight the EMT-related gene TGF-β2 variants in the tumorigenesis of tamoxifen-related UCS, support the possibility that tamoxifen mediates its effect on UCS by enhancing mutations of driver genes, and also provides the rationale for clinical investigation in ER-related gene mutation in breast carcinoma to predict the risk for UCS after tamoxifen treatment.

Keywords: Mutations, TGF, uterine carcinosarcoma

Introduction

Uterine carcinosarcoma (UCS), also known as mixed malignant Müllerian tumor, is a rare (an incidence of 2/100,000), aggressive malignancy, accounting for 2-5% of all uterine malignancies. Approximately 22.5% of UCSs represented a second primary malignancy following breast carcinoma, with an interval of 10-20 years [1], and tamoxifen treatment for breast carcinoma with a positive estrogen receptor (ER) was considered as a risk for UCS [2-4]. Tamoxifen-related UCS accounts 8% of UCS [5], and has comparable stage-specific survival outcomes compared to tamoxifen-unrelated UCS [6]. Histological characteristic of UCS demonstrates histologically both malignant epithelial (carcinoma) and mesenchymal (sarcoma) components. Although the tumorigenesis of UCS remains controversial, increasing evidence supports the origin of both mesenchymal and epithelial components from a common epithelial element that undergoes sarcomatous dedifferentiation [7]. Recently, exome sequencing of uterine and ovarian carcinosarcomas revealed histone genes in epithelial-mesenchymal transition (EMT) for sarcomatous transformation, such as genes encoding histone H2A and H2B, and histone methyltransferase MLL3 [8, 9]. In this study, we show that the EMT-related genes transforming growth factor beta 2 (TGF-β2) were recurrently altered in UCSs after tamoxifen treatment for breast carcinomas, which harbor the ER-related gene E1A binding protein p300 (EP300) or the estrogen receptor 1 (ESR1) mutation. This study reveals the EMT-related gene variant in the pathogenesis of tamoxifen-related UCS and also provides the

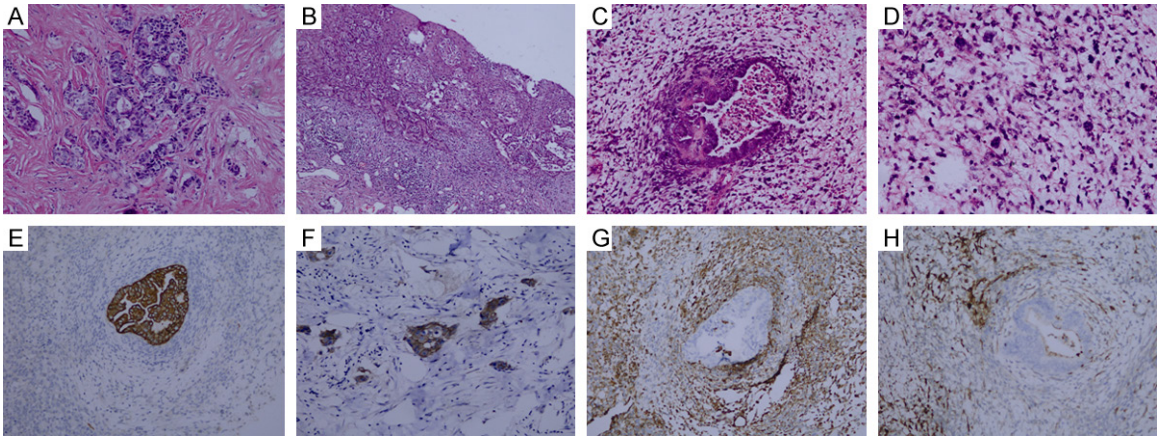

Figure 1. Histopathological and immunohistochemical features of breast carcinoma, Paget's disease and uterine carcinosarcoma for case 2. (A) Invasive ductal carcinoma of breast, HE, × 200; (B) Mammary Paget's disease, HE, × 100; (C) The carcinosarcoma is composed of two components including carcinoma and sarcomatous components, HE, × 200; (D) Enlarged present for sarcomatous component, HE, × 400. (E-H) The positive immunoreactivity for CK20 (E) and c-erbB-2 (F) were observed only in the carcinoma component, while Vimentin (G) and CD10 (H) were expressed only in the sarcomatous component, × 200.

rationale for clinical investigation in ER-related gene mutations in breast carcinoma to predict the risk for UCS.

Materials and methods

Study approval

The study procedure was approved by the Institutional Ethics Board of Shantou University Medical College.

Tissue microdissection

Tumors were microdissected to remove contaminating normal tissue. Normal uterine smooth muscle tissues were collected from both cases to serve as normal comparators in genomic analyses. Genomic DNA was extracted using a GeneRead DNA FFPE Kit (catalog no. 180134; Qiagen GmbH, Hilden, Germany).

Exome sequencing and bioinformatics

Genomic DNA was captured on the Agilent SureSelect Human All ExonV6 human exome array and sequenced using a PE150 sequencer (Illumina Inc, San Diego, CA, USA) as already described [8]. Only the rare, most damaging (nonsynonymous and nonsense) mutations and indels (deletions and insertions) were filtered. Pathway analyses for Gene Ontology (GO) term enrichment were performed using DAVID v6.8 (Database for Annotation, Visualization and Integrated Discovery).

Results

We previously presented a patient (Case 1) with synchronous UCS and contralateral breast carcinoma after tamoxifen therapy [10]. Recently, we identified a postmenopausal woman (Case 2) age 74 with a complaint of vaginal bleeding and low abdominal pain over 5 days. She had a history of invasive ductal carcinoma of breast and mammary Paget's disease previously treated with tamoxifen daily for 6 years. There was a palpable pelvic mass prolapsed into the vagina and some vaginal discharge noted on the vaginal examination. Ultrasonography was significant for a hypoechoic nodule in the uterine cavity. She underwent a total abdominal hysterectomy, bilateral salpingo-oophorectomy after a comprehensive examination. Histopathology finally revealed a uterine heterogonous carcinosarcoma, and this was confirmed by the immunohistochemical staining results, including a strong positivity for CK20 and negative for CD10 or vimentin of the carcinoma component, whereas strongly positive for CD10, vimentin and negative for CK20 of the sarcomatous element (**Figure 1**). The disease stage IB was identified according to the International Federation of Gynecology and Obstetrics (FIGO) classification for this UCS. We therefore took advantage of patient-derived tumors of breast carcinomas and UCSs from these two cases to investigate the genetic alterations in the tamoxifen-related USCs and breast carcinomas.

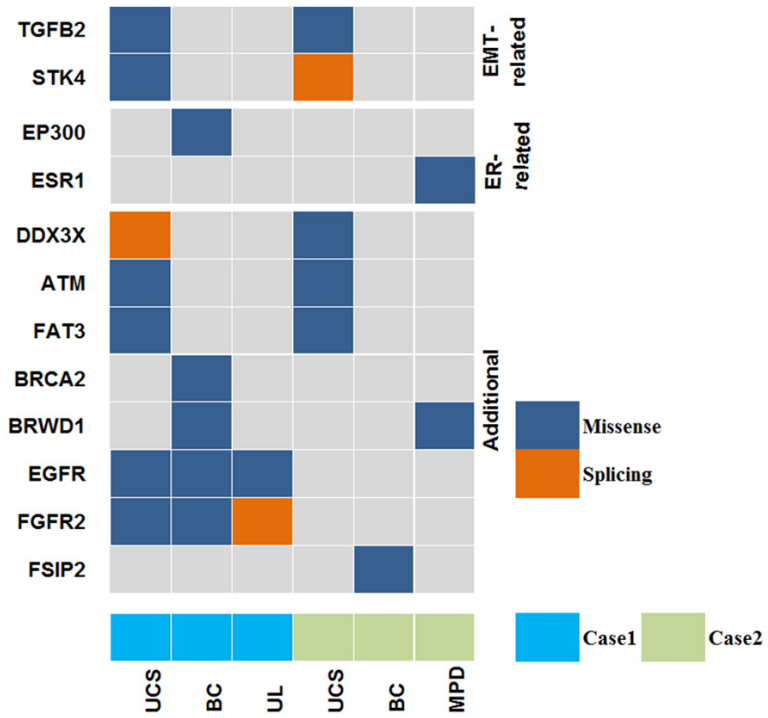

Figure 2. Recurrently mutated genes across uterine carcinosarcomas. The matrix represents individual mutations in tumors, color-coded by type of mutation. Only the most damaging mutation is shown. Genes are classified into functional categories displayed on the right. UCS: uterine carcinosarcoma; BC: breast carcinoma, UL: uterine leiomyoma; MPD: mammary Paget's disease.

We collected formalin-fixed, paraffin-embedded tissue specimens of breast ductal carcinoma, UCS, and leiomyoma from case 1, and breast ductal carcinoma, mammary Paget's disease, and UCS from case 2 for the whole-exome sequencing (WES). The WES yielded a mean depth of 75-fold and more than 85% of the captured exome target region was covered by at least 10 high-quality reads in all samples. Overall, we identified 374 somatic variants in 141 genes shared across the two UCSs, but shared somatic variations across the breast carcinomas. Analysis of these shared genes demonstrated marked enrichment in pathways involved in MAPK including TGF-β2 and serine-threonine kinase-4 (STK4).

We first identified recurrent mutations in the TGF-β2 gene (c. 1039G > A, p.A347T for case 1; c. 1040C > T, p.A347V for case 2, respectively), which is an EMT-related gene that had not been previously implicated in carcinosarcomas (**Figure 2**). The MAPK pathway contributed to TGF-β2-induced EMT in lens epithelial cells [22], and oxidative stress mediates the conversion of endothelial cells into myofibroblasts [23]. In our study, recurrent somatic mutations (c. 550G > A; p.V184M, and c. 1420C > A; p.L474M for case 1; splice site 526-1G > A for case 2) in STK4 were identified in UCSs as well. STK4, also named mammalian sterile STE20-like kinase 1 (MST1), is an upstream kinase of the JNK and p38-MAPK pathways whose expression induces apoptotic morphological changes such as nuclear condensation [24]. Interestingly, recent research found STK4 as a negative feedback for the TGFβ1 signal [25]. Therefore, the interplay between TGF-β2 and STK4 should be to elucidate tumorigenesis.

Unlike in previous exome-sequencing studies of UCS [8, 9], TP53 mutations were not confined to UCS, but ATM, a key molecule that activates p53 after DNA damage; this occurred in both UCSs and was found in our study. We also highlighted some mutations shared between UCS and breast carcinoma and uterine leiomyoma in case 1, such as EGFR and FGFR2. Recurrent mutation of FGFR2 in UCS had been described previously [26].

Tamoxifen is a selective ER modulator that antagonizes the ER in breast tissue and remains a first-line adjuvant treatment for premenopausal breast cancer patients who are ERα positive. We also sought to discover a somatic mutation potentially linked to UCSs and a predictor of resistance to the ER inhibitor tamoxifen in breast carcinoma. We identified the ER-related genes in breast carcinoma, including EP300 (c. 47C > T; p.P16L) for case 1 and ESR1 (c. 1063G > A; p.V355I) for case 2.

Discussion

TGF-β acts as a potent driver of EMT, which has been shown to confer malignant properties [11, 12]. TGF-β signaling was activated in UCS [13].

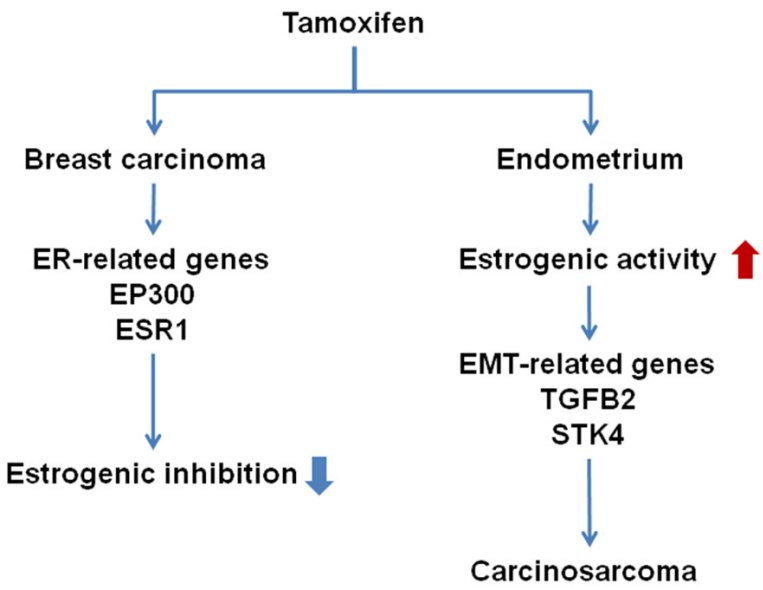

Figure 3. Proposed Model of the role of mutational signaling of EMT- and ER-related genes in UCS development.

Previously, Inoue et al. revealed TGF-β1 mediated EMT process in sarcomatous differentiation driven from carcinomatous components in UCS [14]. Fan et al. identified TGF-β1-mediated ER-induced EMT [15]. To the best of our knowledge, this is the first time anyone has provided evidence of a recurrent TGF-β2 mutation in UCS. Because TGF-β acts as an oncogenic factor, abnormalities in TGF-β signaling result in carcinogenesis [16]. TGF-β1 and TGF-β2 share the same receptors TbetaRI and TbetaRII and the capacity to induce EMT in epithelial cells through the TGF-β/SMAD pathway [12]. We mapped mutations p.A347T and p.A347V in TGF-β2 onto the crystal structure (**Figure 3**), and found two mutations occurred at site of residue 347, which is binding to its receptors [17]. Currently, the theory of tamoxifen's mechanism in endometrial cancer is that tamoxifen appears to mediate its effect on endometrial cells through estrogenic and non-genomic pathways, rather than introducing a genomic alteration as a carcinogen [18]. This is based on the fact that EMT was involved in sarcomatous transformation during UCS development [8]. Evidence of somatic variations in TGF-β2 in our study support the possibility that tamoxifen increases the risk for UCS by enhancing mutations in the driver genes. To date, adjuvant chemotherapy appears to be effective to control recurrence in stage I UCS after surgery [19, 20]. However, a clinical trial of the VEGFR inhibitor pazopanib indicated minimal activity as a second or third line treatment for advanced UCS [21]. Therefore, we purpose that, in addition to being potentially involved in UCS development, TGF-β2 inhibitors, such as the TGFβR-I inhibitor galunisertib might be an alternative targeted therapy approach in patients affected by USC.

The use of tamoxifen in breast cancer patients resulted in an 7.5-fold increased risk of endometrial tumors [27]. The ESR1 gene encodes the estrogen receptor-α (ER-α). ESR1 mutations have been identified in approximately 20% of patients with metastatic ER-positive breast carcinomas who received endocrine therapies, such as tamoxifen and aromatase inhibitors. Mutations in ESR1 were gain-of-function mutations and were clustered in a 'hotspot' within the ligand-binding domain (LBD) of the ER and lead to ligand-independent ER activity [28]. Therefore, the ESR1 mutation was considered as a potential biomarker in acquired endocrine therapy resistance [29, 30]. Based on the fact that ESR1 mutation frequencies were lower in primary breast carcinoma than in metastases, this suggests that in some tumors rare ESR1-mutant clones are enriched by endocrine therapy [31]. In our study, a new LBD-localized ESR1 mutation (V355I) was identified in Paget's disease for case2. EP300, as a "secondary" co-activator, combining with ERα and steroid receptor co-activator protein SRC to form a ERα-SRC-3-EP300 coactivator complex bound to DNA [32]. ASC1 enhances association of EP300, SRC1 at promoters of ERα target genes, and this could be abrogated by tamoxifen [33]. EP300 could redistribute from non-ER enhancers to ER enhancers after E2 treatment and could contribute to resistance to the Bruton's tyrosine kinase (BTK) inhibitor [34, 35]. Therefore, mutations in ESR1 and EP300 may be potential markers in breast carcinoma to predict the tamoxifen resistance, even the risk of UCS.

Combining the evidence from breast carcinoma and UCS, we speculate ER-α is the link between tamoxifen-related UCS and breast carcinoma, and the ER-related gene mutation in breast car-

cinoma is the signal for tamoxifen resistance and a potential marker for the tamoxifen-related UCS, which are shared EMT-related gene mutations, such as TGF-β2 (**Figure 3**). This lends support to further studies on whether the TGF-β2 inhibitor increases the anti-tumor efficacy in UCAs. Further studies are required to define the genetic landscape and best therapeutic approaches to tamoxifen-related UCS in a large cohort.

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Disclosure of conflict of interest

None.

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