Original Article
Microarray-based identification of differentially expressed genes in extramammary Paget’s disease

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Abstract: Extramammary Paget’s disease (EMPD) is a rare cutaneous malignancy accounting for approximately 1-2% of vulvar cancers. The rarity of this disease has caused difficulties in characterization and the molecular mechanism underlying EMPD development remains largely unclear. Here we used microarray analysis to identify differentially expressed genes in EMPD of the scrotum comparing with normal epithelium from healthy donors. Agilent single-channel microarray was used to compare the gene expression between 6 EMPD specimens and 6 normal scrotum epithelium samples. A total of 799 up-regulated genes and 723 down-regulated genes were identified in EMPD tissues. Real-time PCR was conducted to verify the differential expression of some representative genes, including ERBB4, TCF3, PAPSS2, PIK3R3, PRLR, SULT1A1, TCF7L1, and CREB3L4. Generally, the real-time PCR results were consistent with microarray data, and the expression of ERBB4, PRLR, TCF3, PIK3R3, SULT1A1, and TCF7L1 was significantly overexpressed in EMPD (P<0.05). Moreover, the overexpression of PRLR in EMPD, a receptor for the anterior pituitary hormone prolactin (PRL), was confirmed by immunohistochemistry. These data demonstrate that the differentially expressed genes from the microarray-based identification are tightly associated with EMPD occurrence.

Keywords: Extramammary Paget’s disease, differentially expressed genes, microarray-based identification

Introduction
Extramammary Paget’s disease (EMPD) accounts for approximately 6.5% of all Paget’s disease and is a rare cutaneous carcinoma of epidermal origin and glandular differentiation associated with internal malignancies [1]. Because of its not common occurrence, a poor prognosis was presented for EMPD, which is more common in elderly patients [2].

The EMPD is associated with an underlying in situ or invasive neoplasia in 32% of the reported cases. The preferred location for EMPD is wherever apocrine odorous and sweat glands are found. The anogenital and vulvar regions are the most common sites, followed by axillae, penis, and, less frequently, the eyelids, umbilicus, and groin [3]. Tumor cells of EMPD originate either from the intraepidermal cells of apocrine gland ducts or from pluripotent keratinocyte stem cells [4-6]. This view is supported by the fact that EMPD is always at least two different pathologic processes that have common clinical and histologic features characterized by Paget’s cells with a characteristic distribution of cells in a pagetoid pattern [1].

The treatment for noninvasive EMPD is wide surgical excision. In cases of invasive EMPD, abdominoperineal resection or the combined modalities of chemoradiotherapy can be attempted [7-10]. Although the conservative surgery of localized EMPD has the advantage of clinical results, but it harbors a significant risk of recurrences. Therefore, long-term follow-up is required for developing diagnosis and prognosis of EMPD, which remains poor, at earlier stages.

At present, the diagnosis of EMPD is based on uncharacteristic clinical findings in typical locations, deficient changes in response to external therapy, and invasive growth requiring biopsy by histopathology, histochemistry, and electron microscopy [11]. However, molecular characterization of EMPD is very limited, through the use of Cytokeratin immunohistochemical staining
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has been suggested for diagnosis of Paget’s disease [12]. In recent years, multiple genes, such as RCAS1, erbB-2, CDX2, androgen receptors, were frequently expressed in EMPD tissues [13-17]. However, because of limited experience, none of definite markers has been established for EMPD diagnosis and prognosis. Thus, the clinical status for EMPD patients warrants a thorough search for associated genes differentially expressed in EMPD tissues.

Herein, a total of 6 EMPD specimens and 6 normal scrotum specimens were collected for microarray-based identification of differentially expressed genes, some of which were confirmed by real-time PCR and immunohistochemistry (IHC) analysis. The present study provides guidelines for clinical diagnosis of EMPD with specific molecular markers.

Materials and methods

Patients and tissue samples

All subjects were collected from EMPD patients and healthy donors in Huashan Hospital. All EMPD specimens were freshly frozen and identified by HE staining and immunohistochemistry. The conditions of 6 EMPD patients and 6 normal donors subjected for microarray analysis were summarized in Figure 2A. 15 EMPD specimens and 15 control samples (normal scrotum epithelium) were collected for real-time PCR assays. Another 6 EMPD samples and 6 normal control samples were subjected to immunohistochemistry with anti-PRLR antibodies. Informed consent was obtained from each patient or healthy donors prior surgery. This study was approved by Huashan Hospital Clinical Research Ethics Committee.

RNA extraction and purification

Total RNA was extracted and purified using mirVana™ RNA Isolation Kit (Ambion, Austin, TX, US) following the manufacturer’s instructions and checked for a RIN number to inspect RNA integrity by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US).

RNA amplification and labeling

Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Agilent technologies, Santa Clara, CA, US), following the manufacturer’s instructions. Labeled cRNA were purified by RNeasy mini kit (QIAGEN, GmbH, Germany).

Hybridization

Each Slide was hybridized with 1.65 μg Cy3-labeled cRNA using Gene Expression Hybridization Kit (Agilent technologies, Santa Clara, CA, US) in Hybridization Oven (Agilent technologies, Santa Clara, CA, US), according to the manufacturer’s instructions. After 17 hours hybridization, slides were washed in staining dishes (Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Agilent technologies, Santa Clara, CA, US), followed the manufacturer’s instructions.

Data acquisition

Slides were scanned by Agilent Microarray Scanner (Agilent technologies, Santa Clara, CA, US) with default settings, Dye channel: Green, Scan resolution =5 μm, PMT 100%, 10%, 16 bit. Data were extracted with Feature Extraction software 10.7 (Agilent technologies, Santa Clara, CA, US). Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent technologies, Santa Clara, CA, US).

Real-time PCR

RNA was reversely transcribed using SuperScript III Reverse Transcriptase (Invitrogen, CA, US). Real-time PCR was performed according to the manufacturer’s protocol that is amplified with SYBR Green real-time PCR master mix (Sigma, CA, US). Relative expression level of target genes was normalized according to GAPDH. Primer sequences are: ERBB4 forward, 5'-CTGTAATGGCACCCTAGC-3' and reverse, 5'-GTTTGGGTTGTCTCGCATA-3'; TCF3 forward, 5'-TGGTCTGCTCATAGAATTC-3' and reverse, 5'-GAGGCACATAGACAC-3'; PAPSS2 forward, 5'-GCTCTTTGCTGTGGTCAT-3' and reverse, 5'-GTTCTAGTCCTTGCTAGT-3'; PIK3R3 forward, 5'-CATTTGAGAGTGTTGACAGTAT-3' and reverse, 5'-GGCCCAGTGGATGATG-3'; PRLR forward, 5'-TCTCCACCTACCTGATTG-3' and reverse, 5'-CTGAACTCTTTGCTGACCC-3'; SULT1A1 forward, 5'-CCAAGCGGCTCAAGAATAAA-3' and reverse, 5'-GAACTCTGGTCTGAGTATT-3'; TCF7L1 forward, 5'-TCTCTCTACCTCTGTATTG-3' and reverse, 5'-GAAAATCAAGCTCGTACC-3'; CREB3L4 forward, 5'-TCCTCGGTCCTGAC-3' and reverse, 5'-GGCCACGTGGATGATG-3'; PRLR forward, 5'-TCTCCACCTACCTGATTG-3' and reverse, 5'-CTAAGCTGGTTGACTG-3'; SULT1A1 forward, 5'-CCAAGCGGCTCAAGAATAAA-3' and reverse, 5'-CTGAACTCTTTGCTGACCC-3'; SULT1A1 forward, 5'-CCAAGCGGCTCAAGAATAAA-3' and reverse, 5'-CTGAACTCTTTGCTGACCC-3'; GAP-
**Figure 1.** Identification of EMPD. All EMPD samples and normal scrotum epithelium were subjected to H&E staining. Normal epithelium was presented in (A) and enlarged view of indicated field was shown in (B). A representative HE staining result for an EMPD specimen was presented in (C, D). Paget cells with atypical round or oval nuclei and abundant basophilic, amphophilic or clear cytoplasm. (E, F) EMPD sections were subjected to IHC with anti-CK7 antibodies.

DH forward, 5'-TGTGGCCATCAATGACCCCTT-3' and reverse, 5'-CTCCACGACGTACTCAGCG-3'.

**Immunohistochemistry**

Paraffin sections were cut from each block and stained by the biotin-streptavidin-peroxidase method as described previously [18]. The primary antibodies used were anti-PRLR (1:100, Santa Cruz Biotechnology) and anti-CK7 (1:200, Vector Laboratories).

**Statistical analysis**

Student's t test was used for data analysis. A p value of <0.05 (*) was considered statistically significant.

**Results**

**Pathological diagnosis of EMPD**

All samples used in this study were characterized by H&E staining before subjecting to micro-
array, real-time PCR and IHC analysis. Significantly different from normal scrotum epithelium (Figure 1A, 1B), all cases of EMPD sections showed Paget cells with atypical round or oval nuclei and abundant basophilic, amphophilic or clear cytoplasm. The morphological appearance of the Paget cells in EMPD was present as single cells or small clusters and located throughout all layers of the epidermis (Figure 1C, 1D). The cases of EMPD were restricted to the epidermis or adnexal structures in all studied cases. To further verify our HE results, IHC was conducted to check CK7 expression in EPMD tissues. As shown in Figure 1E, 1F, CK7 was specifically expressed in these cells with carcinoma characteristics.

Microarray-based identification of differentially expressed genes in EMPD specimens

To identify specific markers for EMPD patients, microarray was performed to screen differentially expressed genes between 6 EMPD samples and 6 control samples as summarized in Figure 2A. Dot plot analysis was conducted to identify differentially expressed genes (fold change >2) (Figure 2B). A total of 799 up-regulated genes and 723 down-regulated genes were identified in EMPD tissues compared with normal control samples (Table S1). The differential expression pattern of these genes were presented as heatmap in Figure 2C. Gene-ontology (GO) analysis demonstrates that the upregulated genes in EMPD tissues were mainly related to cell cycle, cell division and tumorgenesis, which might be related to deregulated cancer cell growth. While the downregulated genes were mainly associated with epidermal development (Figure 2D) because of the invasion into epidermal tissues by EMPD cells. Moreover, KEGG-pathway analysis showed that the upregulated genes in EMPD were related to pathways in cancer and basal cell carcinoma and that the decreased genes were associated with calcium signaling, endocytosis, and Wnt signaling pathways (Figure 2E). These data suggest that carcinomagenesis-related pathways were activated and normal physiological processes in epidermal tissues were suppressed during EMPD development.

Real-time PCR verification of highly expressed genes in EMPD samples

In view financial limitations, only 6 EMPD samples were subjected to microarray analysis. To further verify the overexpression of these highly expressed genes in EMPD patients, another 15 EMPD samples and 15 control epithelium tissues were collected for real-time PCR analysis. Among the 799 up-regulated genes, many cancer-related genes highly expressed in EMPD specimens were observed, which have not been addressed previously. Moreover, the heat mapping of some classical cancer related gene expression (ERBB4, TCF3, PAPSS2, PIK3R3, PRLR, SULT1A1, TCF7L1, and CREB3L4) showed that these genes were highly expressed in EMPD patients (Figure 3A), suggesting that these genes might be good candidates for EMPD diagnosis biomarkers. As shown in Figure 3B, the expression of ERBB4, PRLR, TCF3, PIK3R3, SULT1A1, and TCF7L1 were significantly higher in EMPD samples than normal control tissues. These data were generally consistent with microarray data from 6 EMPD patients. More importantly, the 6 genes, including PRLR, TCF3, ERBB4, PIK3R3, SULT1A1, and TCF7L1, showed much higher expression levels in the majority of EMPD patients (>80%), highlighting their clinical significance as novel molecular markers in EMPD diagnosis.

PRLR is highly expressed in EMPD tissues

Among our newly identified possible EMPD markers, PRLR, a prolactin receptor highly associated with breast cancer and a possible therapy target for breast cancer [19-22], is preferentially investigated. We performed IHC experiments to analyze PRLR protein expression levels with anti-PRLR antibodies in 6 EMPD specimens as well as normal scrotum tissues from 6 healthy donors. Our result showed that PRLR was specifically expressed in EMPD tissues, especially in Paget cells. Moreover, PRLR was mainly located in cell membrane, consistent with a previous report [23]. However, PRLR was hardly to be detected in normal epithelium tissues. Combining the results in Figure 3A, it demonstrates that both mRNA (9 out of 12 cases) and protein (5 out of 6 cases) of PRLR is overexpressed in EMPD tissues. Thus, PRLR might be used as diagnosis markers in future EMPD treatment.

Discussion

EMPD is a rare cutaneous malignancy and the rarity of this disease has caused difficulties in its characterization. Controversies exist in the reported cases regarding the prevalence of
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Figure 2. Microarray analysis of differentially expressed genes in EMPD. A. The conditions of 6 EMPD patients and 6 normal donors were summarized. B. Dot plot analysis of differentially expressed genes between EMPD tissues and normal scrotum epithelium. C. Heatmapping of differentially expressed genes in EMPD comparing to normal control group. D. Gene-ontology analysis of differentially expressed genes in EMPD patients. E. KEGG-pathway analysis of differentially expressed genes in EMPD samples. Up, upregulated genes; down, downregulated genes.
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concurrent underlying adenocarcinoma or invasive EMPD, associated malignancies, and optimal treatment. Moreover, surgery is still considered the main strategy of treatment for patients with EMPD, although many therapeutic modalities have been attempted on EMPD patients to reduce the significant morbidity. These facts lead to strong need for systematical analysis of differentially expressed genes in EMPD, which might be associated with EMPD progression.

In the present study, microarray was conducted for the first time in EMPD research field to analyze the differential expression between EMPD samples and control epithelium. Using this unbiased strategy across the whole transcriptome, more wide and accurate differentially expressed genes were identified in EMPD patients. A total of 1522 differential genes were primarily found in EMPD specimens (Figure 2C). Though only 6 EMPD cases were used in this study, but the strict screening conditions will partially supplement this shortcoming and make the results more reliable. At least, it could provide hints for identifying significant differential genes or markers for EMPD patients.

In previous studies, the deregulated gene expression associated with EMPD has been found relying on the previous reports about the correlation between these genes and other kinds of carcinomas. As summarized here, the first kind of EMPD-associated gene expression is the overexpression of various cancer-related genes, such as CK7, AR, erbB-2, Cyclin D1, COX-2, IGF-1R, Bcl-xl, HSP105, SP1, VEGF, 5alpha-reductase, p53, Stat5a, BMP7, E-cadherin, hTERT, HPK1, NGF, BDNF, TrkA, TrkB, CXCL17, CXCL4 and c-Myc [13, 14, 24-32]; the second...
kind of EMPD-associated expression is the downregulation of several genes, such as DLC1, CDH1, Wnt-5a, and p63 [29, 33, 34]; the third kind of EMPD-associated alterations is the enhanced phosphorylation of some proteins, including p38, NFkappaB, AKT, ERK, FAK, ATF2, Stat3, and MKK4 [13, 35-38]. In our microarray data, multiple deregulated genes (such as CK7, CXCL17, CXXC4, and BMP7) were also observed, proving our results being reliable. Moreover, several additional cytokeratin proteins, such as CK18, CK19, and CK8, were revealed to be overexpressed in EMPD specimens. Although Wnt-5a was not in the downregulated gene list, other Wnt family members (Wnt7a, Wnt4, and Wnt3a) were observed to be decreased in EMPD samples. It strongly suggests that Wnt signaling pathway might be inactivated in EMPD development (Figure 2E), which is supported by the report that beta-catenin is not activated in EMPD [17].

According to our microarray results (Figure 2C), several upregulated genes were chosen as EMPD-associated gene candidates for further confirmation by real-time PCR analysis. As expected, ERBB4, PRLR, TCF3, PIK3R3, SULT1A1, and TCF7L1 were significantly overexpressed in EMPD samples than normal scrotum epithelium (Figure 3). Without exceptions, these highly expressed genes in EMPD were tightly associated with other kinds of cancers. PIK3R3 induces epithelial-to-mesenchymal transition to promote colorectal cancer metastasis and has been identified as a potential therapeutic target in epithelial ovarian cancer [39, 40]. SULT1A1 polymorphisms are associated with multiple cancer risks [41, 42]. TCF3

Figure 4. Immunohistochemistry (IHC) analysis of PRLR expression in EMPD. IHC was performed with anti-PRLR antibodies in normal epithelium sections and EMPD tissue sections. 2 representative normal epithelium (A, B) and 2 representative EMPD specimens (C, D) stained by IHC were presented. Strong PRLR expression signals were observed in EMPD tissues (mainly in Paget cells) but bot control samples.
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(also named as FGF12) and TCF7L1 also act as a positive regulator in cancer cell growth and initiation [43, 44]. In this study, we first reveal the connection between these cancer-related genes and EMPD occurrences and highlight the clinical significance of these overexpressed genes.

In view of the importance of Peget cells in EMPD progression and unclearness of the origin of EMPD cancer cells, identifying specific markers for Peget cells becomes very essential. Immunohistochemical staining is being used to identify Peget cells. The unique histopathological pattern produced by the intraepidermal infiltration of Peget cells has led to the recognition of the pagetoid spread pattern in other pathologic conditions. Here IHC staining was also performed and a specific EMPD marker PRLR was characterized in over 83% EMPD patients (Figure 4). PRLR, a Prolactin (PRL) transmembrane receptor, interact with PRL to activate the downstream signaling networks in breast cancer [21]. Furthermore, the genetic variations of PRLR are associated with breast cancer risk [20, 22]. We demonstrate that PRLR is overexpressed in EMPD carcinoma cells, and the possibility that PRLR overexpression is resulted from its mutations, is valuable to be investigated in the future. Given that blockade of the PRLR pathway can be considered as a novel antihormonal approach for the treatment of breast and prostate cancer [19], targeting the PRL-PRLR axis may represent an unexploited avenue for therapeutic intervention for EMPD patients.

In summary, the mechanisms underlying progression, invasion, and recurrence of EMPD are largely unknown, possibly involving multiple signaling pathways. Microarray-based screening for deregulated genes in EMPD helps to under the molecular alterations in EMPD patients and provides hints for identifying therapy target, such as PRLR, in EMPD treatment. It needs larger number of EMPD subjects to further confirming the present findings in the future.

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Disclosure of conflict of interest

None.

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