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Comprehensive evaluation of caspase-14 in vulvar neoplasia: An opportunity for treatment with black raspberry extract

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HIGHLIGHTS

- Caspase-14 has been shown to have a role in epithelial differentiation.
- Caspase-14 expression decreases in premalignant lesions and vulvar squamous cell carcinoma.
- Treatment with black raspberry extract increases caspase-14 expression, making it a candidate for topical treatment of vulvar lesions.

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ABSTRACT

Objective. The aim of this study is to determine the expression of caspase-14, a key protein in maturation of squamous epithelia, in archival malignant and premalignant vulvar squamous lesions and examine in-vitro effects of a black raspberry extract (BRB-E) on a vulvar squamous cell carcinoma (VSCC) cell line.

Methods. VSCC cell cultures were exposed to different BRB-E concentrations and used to create cell blocks. Immunohistochemistry for caspase-14 was performed on cell block sections, whole tissue sections, and a tissue microarray consisting of normal vulvar skin, lichen sclerosus (LS), classic and differentiated vulvar intraepithelial neoplasia (cVIN and dVIN respectively), and VSCC.

Results. LS demonstrated abnormal full thickness (5/11) or absent (1/11) caspase-14 staining. dVIN often showed markedly reduced expression (4/7), and cVIN occasionally demonstrated either absent or reduced caspase-14 (6/22). VSCC predominantly had absent or markedly reduced caspase-14 (26/28). VSCC cell cultures demonstrated a significant increase in caspase-14 ($p = 0.013$) after BRB-E treatment: 7.3% (±2.0%) of untreated cells showed caspase-14 positivity, while 21.3% (±8.9%), 21.7% (±4.8%), and 22.6% (±5.3%) of cells were positive for caspase-14 after treatment with 200, 400, and 800 μg/mL BRB-E, respectively. Pair-wise comparisons between the treatment groups and the control demonstrated significant differences between no treatment with BRB-E and each of these treatment concentrations (Dunnett's adjusted *p*-values: 0.024, 0.021, and 0.014, respectively).

Conclusions. Caspase-14 is frequently decreased in premalignant and malignant vulvar squamous lesions, and is upregulated in VSCC cell culture by BRB-E. BRB-E should be further explored and may ultimately be incorporated in topical preparations.

Introduction

Squamous cell carcinoma represents a majority of primary vulvar malignancies [1]. Along with its various precursor lesions, it poses a significant clinical problem. Surgery is a mainstay of treatment but it may be mutilating, affect quality of life, and be followed by local recurrences. However, the vulva is also amenable to topical treatments. The abnormal epidermal differentiation that defines squamous lesions may be a target for cancer chemoprevention and treatment strategies, including the use of natural compounds. In fact, numerous authors have reported on the ability of natural, fruit-derived compounds to inhibit tumorigenesis and promote

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apoptosis in various in vitro models [2–7]. Patient trials at our institution are also underway, including early randomized clinical trials using black raspberry-derived products in oral premalignant squamous lesions, with early success reported [8–10].

Normal epidermal differentiation is characterized by a series of morphological and biochemical events as cells progress from the germinative basal layer to the superficial cornified layer. Caspase-14 is not normally detected in the basal layer but nuclear and cytoplasmic caspase-14 is expressed with increasing intensity as the epithelium matures from the suprabasal layer to the stratum corneum of normal skin [11–13]. This pattern of expression is consistent with a role in epithelial differentiation. Moreover, unlike apoptotic caspases, caspase-14 is largely limited to the epidermis [11]. More recently caspase-14 has been demonstrated to be responsible for the correct processing and degradation of filaggrin, a monomeric structural protein responsible for keratin binding at the junction of the stratum granulosum and stratum corneum. Filaggrin malfunction secondary to caspase-14 absence leads to reduced skin hydration levels, increased water loss and increased UVB photodamage in mouse models [14].

Reduced or low-level caspase-14 expression has been reported in atopic dermatitis [15], psoriasis [16], and in squamous cell carcinomas of both the oral cavity and the uterine cervix [17–19]. Our group has previously shown that a black raspberry extract (BRB-E) inhibited cell growth and promoted apoptosis in cervical squamous cell carcinoma cell lines [2] in concert with increases in caspase-14 mRNA and protein expression (unpublished data). Given the many similarities with cervical lesions herein we focus on the expression of caspase-14 in vulvar squamous lesions. We examine vulvar squamous cell carcinomas as well as various precursor lesions where BRB-E could potentially make an impact. Thus, we performed immunohistochemistry (IHC) for caspase-14 on archival paraffin embedded tissues with lichen sclerosus (LS), high grade vulvar intraepithelial neoplasias of classic type (cVIN) and differentiated type (dVIN), and vulvar squamous cell carcinomas (VSCC). Additionally, IHC for caspase-14 was performed on sections of cell blocks from a vulvar cancer cell line that was treated with different BRB-E concentrations.

Materials and methods

Tissues

Vulvar specimens with squamous pathology including LS, dVIN, cVIN and VSCC were identified using the Ohio State University Wexner Medical Center pathology department database. Archived glass slides from forty-eight specimens were pulled and reviewed by a gynecologic pathologist (AAS). Areas presenting clear cut histologic entities were identified and assigned to either whole section or tissue microarray (TMA) based on (1) amount of tissue and/or (2) possibility of studying different entities in a single whole section. Whole sections of one or two paraffin blocks from 25 specimens were used for immunohistochemistry. A TMA was created with 42 2 mm cores from 23 specimens. In addition to areas of histologically normal vulvar skin identified in vulvar specimens with squamous pathology, whole sections of histologically normal skin from 8 reduction mammoplasties were used to unequivocally define normal patterns of protein expression and validate antibody performance.

Immunohistochemical staining procedure for caspase-14

Paraffin embedded tissue was cut at 4 microns and placed on positively charged slides. Slides were then placed in a 60 °C oven for 1 h, cooled, deparaffinized and rehydrated through xylenes and graded ethanol solutions to water. All slides were quenched for 5 min in a 3% hydrogen peroxide solution in water to block endogenous peroxidase. Antigen retrieval was performed by a heat method in which the specimens were placed in a citric acid solution (pH 6.1) for 25 min at 94 °C using a vegetable steamer and cooled in solution for 15 min. Slides were then placed on a Dako Autostainer immunostaining system. A primary antibody to caspase-14 (Thermo Scientific, Rockford, IL, rabbit polyclonal) was diluted 1:200. A labeled streptavidin–biotin complex was used for detection. Slides were then counterstained with hematoxylin, dehydrated through graded ethanol solutions to xylene and coverslipped.

Cell culture

The human VSCC cell line SW 954 [SW-954, SW954] (ATCC® HTB-117™) (Manassas, VA) was grown in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of Fungizone (Life Technologies; Grand Island, NY) under humidified conditions at 37 °C. Subconfluent cells (0.43×10^6 cells/well) in 6-well cluster plates were exposed to black raspberry extract (BRB-E) [2] suspended in Hybri-Max dimethyl sulfoxide (DMSO; Sigma-Aldrich; St. Louis, MO) or DMSO alone for 32 h. A previous work by some of our group describes the derivation of our black raspberry extract from a dedicated Ohio Rubus occidentalis cultivar plot (Dale Stokes Raspberry Farm, LLC; Wilmington, OH) [2]. Whole black raspberries were harvested, freeze-dried, and pulverized to powder. Ethanolic extraction and partitioning were used to derive an anthocyanin-enriched phytochemical residue. BRB-E was suspended in cell culture grade DMSO immediately prior to in vitro use [2]. Also as previously described, analysis of the compounds in BRB-E identified the four major anthocyanins found in whole black raspberries to be most abundant [2,7,20]. Vulvar cells were exposed to BRB-E doses of 0, 50, 100, 200, 400, and 800 μg/mL in triplicate wells. Following BRB-E exposure, SW 954 cells at approximately 90% confluence were harvested using TrypLE (Life Technologies) and resuspended into a SurePath preservative collection vial (BD Company; Franklin Lakes, NJ).

Cell blocks and caspase-14 expression analysis

Post-treatment VSCC cells in SurePath vials were used to create cell blocks. The procedure included a 10 minute, 2150 revolutions per minute centrifugation; addition of eosin, plasma, and thrombin; and further centrifugation at the previously mentioned settings. The resulting clots were fixed in formalin in our clinical 13-hour processing protocol, embedded in paraffin, cut at 4 microns, and placed on positively charged slides. Slides were then stained with caspase-14 using the same process and specifications as the TMA and whole sections (previously described). Microscopic photographs were taken to represent each cell block in its entirety. Blinded to the presence and concentration of BRB-E, a single pathologist (ASJP) manually quantified the number of cells staining positive and the number of cells staining negative in each photographed cell block, using an electronic counter. Positive cells were defined by moderate to strong immunostaining. Weak staining (a faint blush) and no staining were considered negative. Percentage of positive-staining cells in the three cell blocks of each concentration of BRB-E were compared to the control using an analysis of variance model with Dunnett's method for adjusting for multiple comparisons. An adjusted p-value of 0.05 was considered significant.

Additional testing of the SW 954 cell line

The HPV status of the SW 954 cell line was investigated using an immunohistochemical stain for p16 and PCR for high risk HPV types 16 and 18 (supplemental materials and methods, Supplemental Fig. 1).