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High snail expression predicts a poor prognosis in breast invasive ductal carcinoma patients with HER2/EGFR-positive subtypes

Hui-Yu Chang [a, 1], Yu-Kai Tseng [b, c, 1], Yu-Chia Chen [d], Chih-Wen Shu [a], Miaw-I. Lin [e, f], Huei-Han Liou [a], Ting-Ying Fu [g], Yun-Chung Lin [h], Luo-Ping Ger [a, i], Ming-Hsin Yeh [j, k, **], Pei-Feng Liu [a, l, *]

[a] Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan
[b] Department of Orthopedics, Show Chwan Memorial Hospital, Changhua 50008, Taiwan
[c] Department of Orthopedics, National Cheng Kung University Hospital, Tainan 70401, Taiwan
[d] Department of General Surgery, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan
[e] Department of Nursing, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan
[f] Center for Geriatrics and Gerontology, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan
[g] Department of Pathology and Laboratory Medicine, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan
[h] Department of Pathology, China Medical University Hospital, Taichung 40447, Taiwan
[i] Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung 80424, Taiwan
[j] Department of Surgery, Chung Shan Medical University Hospital, Taichung 40201, Taiwan
[k] School of Medicine, Chung Shan Medical University, Taichung 40201, Taiwan
[l] Department of Nursing, Shu-Zen Junior College of Medicine and Management, Kaohsiung 82144, Taiwan

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ABSTRACT

Background: High Snail expression is known as a poor prognostic factor in breast cancer. However, its prognostic impact for breast cancer with different molecular subtypes is still controversial.
Methods: Snail expression was examined by immunohistochemistry in tissue microarray slides of 85 corresponding tumor-adjacent normal (CTAN) and 247 breast invasive ductal carcinoma (IDC) tissues. Multivariable Cox regression analysis was used to assess the impact of Snail expression on survival rate by different molecular subtypes of breast IDC patients.
Results: The level of Snail expression in IDC tumor tissues was significantly higher than that in CTAN tissues. Moreover, high Snail expression had direct impacts on poor disease specific survival (DSS) and disease-free survival (DFS) in breast IDC patients with human epidermal growth factor receptor 2 (HER2)-positive and human epidermal growth factor receptor (EGFR)-positive statuses as well as the HER2 intrinsic subtype. Additionally, breast IDC patients with a combination of three prognostic factors, including high Snail expression and HER2-positive and EGFR-positive statuses, had much poor DSS and DFS with a statistically significant linear trend.
Conclusion: High Snail expression could predict a poor prognosis for breast IDC patients with HER2/EGFR-positive subtypes.

1. Introduction

Breast invasive ductal carcinoma (IDC), a common type of breast cancer, comprises nearly 70–80% of all breast cancer diagnosis. Breast cancer is divided into six intrinsic subtypes, including the luminal A, luminal B, HER2-enriched, basal-like (triple-negative) [1], normal breast and claudin-low [2–4] subtypes. According to immunohistochemistry (IHC) analysis, it is also divided into several molecular subtypes, including estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2),

* Corresponding author. Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan.
** Corresponding author. Department of Surgery, Chung Shan Medical University Hospital, Taichung, Taiwan..
E-mail addresses: breast5321@gmail.com (M.-H. Yeh), pfliu@vghks.gov.tw (P.-F. Liu).
[1] H.Y. Chang and Y.K. Tseng contributed equally to this work.

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Ki-67, human epidermal growth factor receptor (EGFR), and cytokeratin 5/6 (CK5/6) [5]. These subtypes are characterized by different clinicopathological outcomes, prognosis, and treatment responses [3].

Snail is a key regulator of the epithelial-mesenchymal transition (EMT) of tumor cells [6], and it may initiate the EMT process by suppressing *E*-cadherin expression [7]. Importantly, aberrant expression of Snail is associated with mammary tumor recurrence *in vivo* [8] and lymph node metastasis of breast carcinoma [9–11], especially in breast IDC patients [12].

The propensity of breast cancer to give rise to clinical characteristics depends on the interaction of different breast cancer subtypes and protein expression [13]. Thus, evaluating the impact of Snail expression in different breast cancer subtypes is an important task to identify its value as a prognostic biomarker. In the present study, we compared the expression levels of Snail among 85 corresponding tumor-adjacent normal (CTAN) tissues and 247 breast IDC tissues using tissue microarray (TMA) slides. We investigated the impact of Snail expression on the tumorigenesis of 247 breast IDC patients and on the prognosis of 247 breast IDC patients with different breast cancer molecular subtypes. Moreover, the combined expression of Snail and other molecular subtype markers for prognosis of 247 breast IDC patients was also analyzed.

2. Patients and methods

2.1. Tissue specimens

The subject population was a consecutive series of females with non-metastatic breast ductal carcinoma who underwent primary surgical resection in Kaohsiung Veterans General Hospital (KVGH) between 1991 and 1999 [14]. Subjects with a prior history of cancer, bilateral breast tumors, resection with a positive surgical margin or who received any anti-cancer therapy before the operation were excluded. Data on the age at diagnosis, menopause status, primary tumor TNM stage, histologic grade, adjuvant therapy type, and tumor recurrence time and site were collected from charts. The pathological TNM classification determined at the time of the initial resection of the tumor was standardized in accordance with the guidelines of the 2010 American Joint Committee on Cancer system. This study protocol was independently reviewed and approved by the Institutional Review Board at KVGH (Kaohsiung, Taiwan; IRB number: VGHKS12-CT2-07). The requirements for written informed consent from subjects were waived by the Institutional Review Board of the KVGH because all data and specimens were previously collected and analyzed anonymously.

2.2. TMA construction

Initially, representative areas of IDC and CTAN tissues were selected by a senior breast cancer pathologist from hematoxylin-eosin-stained sections of paraffin-embedded tissues. Then, one core with CTAN tissue and two cores with IDC tissue from each patient were punched in TMA. Of 309 IDC patients, 309 CTAN cores and 618 IDC cores were collected in two TMA. After exclusion of missing cores, only 247 IDC patients were stained or scored in this study. Afterward, each TMA block was cut into 4-μm-thick paraffin sections using standard techniques.

2.3. Immunohistochemistry

For IHC staining in this study, the Novolink polymer detection system (Leica, Newcastle Upon Tyne, United Kingdom) and VECTASTAIN® ABC system (Vectoer Laboratories, Burlingame, CA) were used for Snail and the intrinsic subtype markers, respectively. TMA sections were de-waxed in xylene and then rehydrated in a graded alcohol series. Antigen retrieval was performed by immersing the slides in Tris-EDTA buffer (10 mM, pH 9.0) for 10 min at 125 °C in a pressure cooker. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min. Non-specific binding sites were blocked by Protein block (Novolink max polymer detection system) for 30 min. Slides were incubated with anti-Snail goat polyclonal (dilution 1:100; Abcam, Cambridge, MA, USA) antibody in primary antibody diluent (Tris, Green, ScyTek Laboratories, Logan, UT, USA) overnight at 4 °C in a wet chamber. Color was developed with a solution of 0.03% diaminobenzidine for 2 min at room temperature, and the sections were counterstained with hematoxylin.

Immunostaining for ER, PgR, and HER2 was performed using similar procedures as those described above, except a Vectastain ABC kit and sodium citrate buffer (for antigen retrieval; 0.1 M, pH 6.0) were used. In addition, anti-ER mouse monoclonal (dilution 1:200, Leica, Newcastle Upon Tyne, United Kingdom), anti-PgR mouse monoclonal (dilution 1:200, Leica, Newcastle Upon Tyne, United Kingdom), anti-HER2 mouse monoclonal (dilution 1:50, Leica, Newcastle Upon Tyne, United Kingdom), anti-Ki67 mouse monoclonal (dilution 1:300, Dako, Glostrup, Denmark), anti-EGFR mouse monoclonal (dilution 1:50, Leica, Newcastle Upon Tyne, United Kingdom), and anti-CK 5/6 mouse monoclonal (dilution 1:50, Dako, Glostrup, Denmark) antibodies were used. In brief, the intrinsic subtypes of breast cancer were sorted as luminal A when ER and/or PgR-positive, HER2-negative, and Ki-67 < 14%; luminal B when ER and/or PgR positive, HER2 positive or HER2 negative, and Ki-67 ≧ 14%; HER2 when ER, PgR absent, and HER2 overexpressed or amplified, and triple negative when ER, PgR absent, and HER2 negative.

2.4. Immunohistochemical analysis and scoring

Initially, a breast cancer pathologist (Dr. Ting-Ying Fu) accompanied a technician to evaluate the slides until all discrepancies were resolved. Subsequently, the technician independently reviewed all slides, except those cores with incorrect or uncertain contents, which must be scored by the pathologist. Then, 5% of the core samples of each staining intensity were randomly selected for re-evaluation by the above pathologist until all discrepancies were resolved. If the pathologist and technician agreement with respect to IHC scores was <95%, the technician re-reviewed and re-scored all slides again and the pathologist accompanied the technician in reviewing the discrepancies again until another set of random core samples had scoring agreement of 100%. During the evaluation, both evaluators were blinded to the patients' clinicopathologic outcomes. The degree of immune reactivity was scored using a semi-quantitative approach based on the staining intensity and percentage. In brief, the extent of positivity was scored as 0 when the percentage of positive cells was <5%; 1 when it was 5–25%; 2 when it was 26–50%; 3 when it was 51–75%; and 4 when it was >75%. The intensity was scored as 0 when no positive cells were identified, 1 when weak staining was identified, 2 when moderate staining was identified, and 3 when strong staining was identified (Fig. 1A). The extent and intensity scores were added to obtain a total score, which ranged from 0 to 7. Snail protein expression was dichotomized as low expression (≤the cutoff point) and high expression (>the cutoff point) with the cutoff point set at the 34th percentile based on the distribution of the protein score. The cutoff values were 4 for Snail expression in IDC tissues.