RESEARCH ARTICLE

Immunohistochemical Expression of EGFR, NF-κB and Cyclin D1 in Sinonasal Inverted Papilloma and Squamous Cell Carcinoma

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Abstract

BACKGROUND: Sinonasal inverted papilloma (SIP), a benign epithelial growth in the sinonasal region with epidermoid epithelial transformation, has been known for its invasiveness, recurrence, and its link with malignancy. Meanwhile sinonasal squamous cell carcinoma (SSCC) is an epithelial malignancy on squamous cells from the sinonasal region. Epidermal growth factor receptor (EGFR), Nuclear Factor κB (NF-κB), and Cyclin D1 are factors that might play an important role in proliferation of SIP and SSCC. This research was conducted to investigate the expressions of EGFR, NF-κB and Cyclin D1 in SIP and SSCC.

METHODS: A cross-sectional study by examining the EGFR, NF-κB, and Cyclin D1 immunohistochemical expressions of SIP and SSCC was conducted. Subjects whose blocks were used in this research, were diagnosed as SIP and SSCC at the Otorhinolaryngology-Head and Neck Surgery Clinic, Dr. Saiful Anwar General Hospital. Samples were selected, processed for immunohistochemistry, evaluated and statistical analyzed.

RESULTS: Twenty-four SIP and 9 SSCC subjects with their paraffin blocks were selected. Clear immunohistochemical expressions of EGFR, NF-κB, and Cyclin D1 were observed for both SIP and SSCC. Significantly higher immunostaining levels of EGFR (45.6%, p=0.001) and NF-κB (42.2%, p=0.013) were observed in SSCC. Immunostaining levels of EGFR vs. NF-κB were moderately correlated (r=0.437), while the immunostaining levels of NF-κB vs. Cyclin D1 were strongly correlated (r=0.602).

CONCLUSION: Expression of EGFR and NF-κB in SSCC were higher than the EGFR and NF-κB expression in SIP, suggesting that EGFR and NF-κB play an important role in sinonasal malignancy.

KEYWORDS: sinonasal, inverted papilloma, SCC, EGFR, NF-κB, Cyclin D1

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Introduction

Sinonasal inverted papilloma (SIP) is a benign epithelial growth in the nose and paranasal sinus region which are invasive, recurrent, and linked with malignancy.(1) It is one of the 3 Schneiderian papilloma types which is defined by WHO (inverted, fungiform, cylindrical cell type). SIP is a rare tumor with incidence predicted between 0.6-1.5 cases per 100,000 people, and 0.5-4% of all sinonasal tumors.(2) Symptoms are usually not specific and closely resembles the disease of inflammation. The most common symptoms are one-sided nasal blockage, followed by blood-tinged nasal secretion, headache, facial pain, hyposmia, and epiphora, which are occurred months before diagnosed.(3)

Sinonasal squamous cell carcinoma (SSCC) most commonly occurs in males between 50-60 years old. Patients with SSCC visit health providers with non-specific
complaints similar with SIP. SSCC occurs from SIP in 2-27% cases.(4) The sinonasal cavity covered with respiratory epithelium suffers squamous metaplasia that continuous to form squamous intraepithelial neoplasia to SSCC.(5)

Epidermal growth factor receptor (EGFR) is a 170 kDa protein and a member of the ErbB family from receptor tyrosine kinase (RTK) which promotes cell survival, proliferation, invasiveness. EGFR peptides promote the dimerization of EGFR and phosphorylation of tyrosine residue in its cytoplasmic chain and then promotes Protein kinase C (PKC), Phosphatidylinositol 3 Kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR), Src, signal transducers and activators of transcription (STAT), and activates Ras/rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) kinase (MEK)1/ERK1/2.(6) MAPK has been introduced as important protein in inducing proliferation of tumors.(7,8) Increase expression of EGFR from mutation are obtained in 88% of SIP cases and 77% of SSCC.(9)

NF-κB is predicted to be the missing link between inflammation and malignancy.(10,11) NF-κB is a group of dimer protein binding with a similar binding site called κB site. NF-κB promotes some malignancy characteristics including continuous cell proliferation.(12) NF-κB activates Cyclin D1 which promotes cell proliferation through cell cycle modulation. In the early gap (G)1 phase of the cell cycle, cyclin-dependent kinase (CdK)4 and/or 6 are activated by Cyclin D. CdK4/6 with Cyclin D1 complex will initiate phosphorylation from retinoblastoma protein (pRb) during early G1 phase. As a result, the transcription E2F responsive genes are needed in cell cycle progression to S phase to become active.(12)

To our knowledge, investigation in EGFR, NF-κB and Cyclin D1 of SIP and SCC has never been conducted. Therefore, current research was conducted to widen our view about the correlation of these biomarkers with SIP and SCC.

Methods

This research was an analytical observation with cross-sectional approach by examining the EGFR, NF-κB, and Cyclin D1 immunohistochemical expressions of SIP and SSCC. The research protocol was approved by the Dr. Saiful Anwar General Hospital Research Ethic Commission (Number: 400/013/K.3/302/2019).

Sample Collection and Selection

Samples were obtained from the paraffin blocks of biopsies/post-operative tissues. Subjects whose blocks were used in this research, were diagnosed as SIP and SSCC at the Otorhinolaryngology-Head and Neck Surgery Clinic, Dr. Saiful Anwar General Hospital, Malang, Indonesia, in the period of January 1, 2016 to December 31, 2018. Subjects those were treated with radiotherapy and/or chemotherapy, were excluded. The blocks were retrieved from pathology laboratory and processed with immunohistochemistry.

Immunohistochemistry

Paraffin blocks were sliced in 4-μm thickness, deparaffinized and antigen retrieved. After washing with phosphate buffered saline (PBS), the tissue sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were incubated with 2% bovine serum albumin. Then each of the following primary antibodies was applied. For EGFR detection, a mouse monoclonal antibody specific for an epitope mapping between amino acids 1114-1147 within a C-terminal cytoplasmic domain of EGFR of human origin (A-10, Santa Cruz Biotechnology, Dallas, TX, USA) was applied. For NF-κB detection, a mouse monoclonal antibody raised against amino acids 120-239 mapping at the N-terminus of NF-κB p50 of human origin (E-10, Santa Cruz Biotechnology), was applied. For Cyclin D1, a mouse monoclonal antibody raised against amino acids 1-295 representing full length cyclin D1 of human origin (A-12, Santa Cruz Biotechnology), was applied. After the first antibody, N-Histofine High Stain HRP (MULTI) (Nichirei Biosciences, Tokyo Japan) kit was used. The peroxidase activity was visualized by immersing tissue sections in N-Histofine DAB-2V (Nichirei Biosciences), resulting in a brown reaction product. Tissue sections were finally counterstained with hematoxylin and mounted.

Immunohistochemical Evaluation

The EGFR, NF-κB and Cyclin D1 immunohistochemical expressions were examined on 5 fields per slide per subject under a light microscope with 400x magnification by using ImmunoRatio, cross-checked by two trained examiners under supervision of a certified pathologist. Immunostaining levels were delivered in percentage (%) and statistical analyzed with SPSS Statistics, version 17.0 (SPSS Inc., Chicago, IL, USA) for Chi-Square, Mann-Whitney, Spearman-Pearson tests. The p-value<0.05 was considered significant.
Results

Subject Characteristic
Twenty-four SIP and 9 SSCC subjects with their paraffin blocks were selected. General characteristics of subjects can be seen in Table 1. For SIP, most subjects were male, older adult with average age of 50.5 years old, having lower education level, >6-months-to-1-year duration of symptom, and high-risk occupation. Meanwhile, for SSCC, most subjects were also male, older adult with average age of 55.2 years old, and having >6-months-to-1-year duration of symptom. Interestingly, higher number of subjects with higher education level and low-risk occupation was

| Characteristic          | SIP          | SSCC         | P-value |
|-------------------------|--------------|--------------|---------|
| **Gender**              |              |              |         |
| Male                    | 15 (62.5%)   | 8 (88.9%)    | 0.142   |
| Female                  | 9 (37.5%)    | 1 (11.1%)    |         |
| **Age Group**           |              |              |         |
| Younger Adult           |              |              |         |
| 25-35 years old         | 3 (12.5%)    | 1 (11.1%)    | 0.407   |
| 36-45 years old         | 6 (25%)      | 1 (11.1%)    |         |
| Older Adult             |              |              |         |
| 46-55 years old         | 5 (20.8%)    | 2 (22.2%)    |         |
| 56-65 years old         | 8 (33.3%)    | 3 (33.3%)    |         |
| >65 years old           | 2 (8.3%)     | 2 (22.2%)    |         |
| **Education level**     |              |              |         |
| Lower education level   |              |              |         |
| Elementary school       | 11 (45.8%)   | 3 (33.3%)    | 0.697   |
| Junior high school      | 3 (12.5%)    | 1 (11.1%)    |         |
| Higher education level  |              |              |         |
| Senior high school      | 5 (20.8%)    | 2 (22.2%)    |         |
| Bachelor degree         | 4 (16.7%)    | 2 (22.2%)    |         |
| Post graduate degree    | 1 (4.2%)     | 1 (11.1%)    |         |
| **Duration of symptom** |              |              |         |
| ≤3 months               | 6 (25%)      | -            | 0.467   |
| >3 months – 6 months    | 4 (16.7%)    | 3 (33.3%)    |         |
| >6 months – 1 year      | 9 (37.5%)    | 5 (55.6%)    |         |
| >1 year – 2 years       | 1 (4.2%)     | -            |         |
| >2 years – 3 years      | 2 (8.3%)     | 1 (11.1%)    |         |
| >3 years – 6 years      | 1 (4.2%)     | -            |         |
| >6 years – 9 years      | 1 (4.2%)     | -            |         |
| **Occupation**          |              |              |         |
| High Risk               |              |              |         |
| Farmer                  | 8 (33.3%)    | -            | 0.286   |
| Leather factory labor   | 1 (4.2%)     | -            |         |
| Wood factory labor      | 1 (4.2%)     | 1 (11.1%)    |         |
| Miner                   | 2 (8.3%)     | -            |         |
| Driver                  | 1 (4.2%)     | 1 (11.1%)    |         |
| Leather seat maker      | -            | 1 (11.1%)    |         |
| Low Risk                |              |              |         |
| Nurse                   | 1 (4.2%)     | -            |         |
| Merchant                | 2 (8.3%)     | 3 (33.3%)    |         |
| Housewife               | 4 (16.7%)    | -            |         |
| Programmer              | 1 (4.2%)     | -            |         |
| Teacher                 | 2 (8.3%)     | 1 (11.1%)    |         |
| Office worker           | 1 (4.2%)     | 2 (22.2%)    |         |

*Chi-Square Test, SIP: Sinonasal Inverted Papilloma, SSCC: Sinonasal Squamous Cell Carcinoma.
observed in SSCC. In more detailed observation, number of the subjects with education level of elementary school was the highest in both SIP (45.8%) and SSCC (33.3%). Although there were differences in various characteristics for both SIP and SSCC subjects, there was no significant difference based on the statistical analysis.

**EGFR, NF-κB, and Cyclin D1 Immunohistochemical Expressions**

Clear immunohistochemical expressions of EGFR, NF-κB, and Cyclin D1 were observed for both SIP and SSCC (Figure 1). Higher immunostaining levels of EGFR (45.6%) and NF-κB (42.2%) were observed in SSCC, while higher immunostaining levels of Cyclin D1 (30.42%) was observed in SIP (Figure 2 & Table 2). Both the immunostaining levels of EGFR and NF-κB were significantly higher in SSCC, \( p=0.001 \) and \( p=0.013 \), respectively. Based on the correlation test for SIP, the immunostaining levels of EGFR vs. NF-κB were moderately correlated \( (p=0.03, r=0.437) \) (Table 3). In addition, the immunostaining levels of NF-κB vs. Cyclin D1 were strongly correlated \( (p=0.002, r=0.602) \) as well. Meanwhile, the correlation test for SCC did not show any significant correlation.

The average age of SIP (50.5 years old) and SSCC (55.2 years old) subjects of the current study were similar to a previous report with the average age of SIP was 53.72 years old (13), whereas the age of SSCC was between 50 to 60 years old (14). The average duration of symptom in current research were similar with the previous report which was 17.6 months (13). This long duration was caused by unspecific symptoms suffered by the patients.

In the current research, EGFR was overexpressed in both cases, especially in SSCC. This overexpression was found in esophageal SCC as well, about 59.6 to 76% cases (15). There was a moderate positive correlation between immunostaining levels of EGFR and NF-κB in SIP. In oral SCC, it has been reported that there was an increase of NF-κB expression in the higher stage of oral SCC (16). Increase of NF-κB immunostaining levels was also found in current study.

In current research, immunostaining level of Cyclin D1 was a slightly higher in SIP than in SCC (4). This result is in accordance to the previous report showing that expression of
Cyclin D1 in oral cavity with dysplasia was not significantly different from the one with malignancy transformation.\(^{(17)}\) In addition, high expression of Cyclin D1 was reported in low-grade histopathology and early-stage of bladder SCC, whereas low expression of Cyclin D1 were found in high-grade histopathology and late-stage. Low Cyclin D1 was significantly linked with poorer prognosis.\(^{(18)}\)

Cell proliferation also could involve some pathway other than Cyclin D1 via Ras-ERK and PI3K-Akt pathway.\(^{(19)}\) Higher expression of EGFR, NF-κB and Cyclin D1 were related to worse cell differentiation level. Epithel which was organized well in the beginning, lost its characteristics. Thus, it became similar to connective tissue in epithelial-mesenchymal-transition (EMT) which led to malignancy. EGFR was predicted to have a role in initiating EMT related to its mutation.\(^{(20)}\) The increased expression of NF-κB was linked with the activation of the transcription factor of EMT and an increased in Cyclin D1 expression that increased the speed of cell cycle of G1 phase of abnormal cell proliferation.\(^{(21)}\)

Table 2. Comparison of EGFR, NF-κB and Cyclin D1 immunostaining levels of SIP and SSCC. SIP: Sinonasal Inverted Papilloma, SSCC: Sinonasal Squamous Cell Carcinoma.

| Variable | EGFR | Mean (%) | Median | SD  | Minimum | Maximum | *p-value |
|----------|------|----------|--------|-----|---------|---------|----------|
| SIP      | 17.71| 10       | 16.22  | 5   | 5       | 60      | 0.001    |
| SSCC     | 45.56| 45       | 19.6   | 20  | 20      | 75      |          |

*Mann Whitney Test

| Variable | NF-κB | Mean (%) | Median | SD  | Minimum | Maximum | *p-value |
|----------|-------|----------|--------|-----|---------|---------|----------|
| SIP      | 22.5  | 17.5     | 16.94  | 5   | 5       | 80      | 0.013    |
| SSCC     | 42.22 | 40       | 22.79  | 20  | 20      | 80      |          |

*Mann Whitney Test

| Variable | Cyclin D1 | Mean (%) | Median | SD  | Minimum | Maximum | *p-value |
|----------|-----------|----------|--------|-----|---------|---------|----------|
| SIP      | 30.42     | 30       | 20.16  | 5   | 5       | 60      | 0.22     |
| SSCC     | 22.44     | 15       | 23.1   | 2   | 2       | 60      |          |

*Mann Whitney Test
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Table 3. Correlation coefficient of EGFR, NF-κB, and Cyclin D1 immunostaining levels of SIP and SSCC.

| Case   | Variable | Correlation Coefficient (r) | p-value |
|--------|----------|----------------------------|---------|
| SIP    | EGFR vs. NF-κB | 0.437                     | -       | 0.030* |
|        | NF-κB vs. Cyclin D1 | 0.602                     | -       | 0.002* |
|        | EGFR vs. Cyclin D1 | 0.153                     | -       | 0.477  |
| SSCC   | EGFR vs. NF-κB | -                         | 0.375   | 0.32   |
|        | NF-κB vs. Cyclin D1 | 0.504                     | -       | 0.17   |
|        | EGFR vs. Cyclin D1 | 0.539                     | -       | 0.13   |

Conclusion

Expression of EGFR and NF-κB in SSCC were higher than the EGFR and NF-κB expression in SIP, suggesting that EGFR and NF-κB play important role in sinonasal malignancy.

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