

Copy Number Alterations of *BCAS1* in Squamous Cell Carcinomas

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Background: Breast carcinoma amplified sequence 1 (*BCAS1*), located in 20q13, is amplified and overexpressed in breast cancers. Even though *BCAS1* is expected to be an oncogene candidate, its contribution to tumorigenesis and copy number status in other malignancies is not reported. To elucidate the role of *BCAS1* in squamous cell carcinomas, we investigated the copy number status and expression level of *BCAS1* in several squamous cell carcinoma cell lines, normal keratinocytes and primary tumors. **Methods:** We quantitated *BCAS1* gene by real-time polymerase chain reaction (PCR). Expression level of *BCAS1* was measured by real-time reverse transcription-PCR and immunoblot. **Results:** Seven (88%) of 8 squamous cell carcinoma cell lines showed copy number gain of *BCAS1* with various degrees. *BCAS1* gene in primary tumors (73%) also showed copy number gain. However, expression level did not show a linear correlation with copy number changes. **Conclusions:** We identified copy number gain of *BCAS1* in squamous cell carcinomas. Due to lack of linear correlation between copy numbers of *BCAS1* and its expression level, we could not confirm that the overexpression of *BCAS1* is a common finding in squamous cell carcinoma cell lines. However, this study shows that the copy number gain of *BCAS1* is a common finding in squamous cell carcinomas.

Key Words: Gene dosage; DNA copy number variations; *BCAS1* protein, human; Gene expression; Carcinoma, squamous cell

Cancer is a genetic disease. The beginning of cancer stems from genetic changes that are critical in the control of cell cycles. For decades, researchers have investigated genes that promote or suppress the development of cancers. Alterations of many genes in cancers were reported and their functions in carcinogenesis were elucidated. Among the alterations, studies on copy number changes have turned out to be important in understanding pathogenesis and providing better patient care. Sometimes, copy number alterations cause changes in gene activity. As a result, tumor suppressor genes can be inactivated and an oncogene can be activated. These changes may lead to the disruption of the normal cell cycle and a malignant tumor may develop as a result.

Knowing the copy number of a gene in certain tumor types can be valuable in terms of diagnosis, treatment and predicting prognosis of a cancer patient. For example, a 1p deletion in neuroblastoma correlates with an unresectable and metastatic disease;¹ *N-MYC* amplification of neuroblastoma is related to poor

prognosis;² and human epidermal growth factor receptor 2 (*HER-2*) amplification of breast cancer correlates with an aggressive phenotype.³

Among the many copy number alterations reported, chromosome 20q13 is the commonly amplified region in breast cancers.⁴ Several genes, including amplified in breast cancer 1 (*AI-B1*),⁵ breast tumor amplified kinase (*BTAK*),⁶ breast carcinoma amplified sequence 1 (*BCAS1*), *CAS*,⁷ and transcription factor AP-2 gamma (*TFAP2C*),⁸ are located in this region and reported to be associated with various phenotypes. *BCAS1* is one of genes located in the region and is considered to be an oncogene candidate. *BCAS1* is amplified in several breast cancer cell lines and overexpressed in some of the cell lines with amplification. However, how *BCAS1* promotes breast cancer development is not known. Inducing *BCAS1* expression in NIH3T3 cell lines *in vitro* failed to induce cellular transformation.⁹ This is why *BCAS1* is not considered a prototypical oncogene.

Besides breast cancers, amplification of the 20q13 region is

reported in ovarian tumors,¹⁰ colon cancers,¹¹ nasopharyngeal carcinomas,¹² gliomas,¹³ and pancreatic carcinomas.¹⁴ However, alterations of copy number and expression levels of *BCAS1* are not reported in tumors other than breast cancers.

Squamous cell carcinoma (SCC) is one of the common malignant tumors. SCCs can occur in any organ covered by squamous epithelium, such as skin, uterine cervix, lung, esophagus, pharynx and so on. Alterations of certain genes, including tumor necrosis factor receptor superfamily, member 10b (*TNFRSF-10B*),¹⁵ phosphatase and tensin homolog (*PTEN*),¹⁶ inhibitor of growth protein 1 (*ING1*),¹⁷ *LZTS1/FEZ1*,¹⁸ WW domain-containing oxidoreductase (*WWOX*),¹⁹ ring finger protein (C3H2C3 type) 6 (*RNF6*),²⁰ are reported to be associated with SCCs. Genes that show copy number gain in SCCs are SRY-box 2 (*SOX2*),²¹ B-cell lymphoma/leukemia 11A (*BCL11A*), *REL*, *ECT2*, *PI3-KCA*²² and so on. However, the causal relationship between copy number alterations of any gene and SCC development has not yet been established.

To study the contribution of *BCAS1* in the development of SCCs, we need an *in vitro* model that has copy number alterations of *BCAS1* at variable expression levels. In this study, we screened SCC cell lines and primary tumors to evaluate status of copy number and expression of *BCAS1*. This study will provide the foundation to establish an *in vitro* model for *BCAS1* in the study of the carcinogenesis of SCCs.

MATERIALS AND METHODS

Cell culture

Eight SCC cell lines (SNU-017, SNU-703, SNU-1299, HCC-1588, YD-38, YD-8, FaDu, and SiHa) were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea) and cultured as per the recommended protocol set by KCLB. As a negative control of SCC cell lines, Human Epidermal Keratinocytes (HEK1) were purchased from Invitrogen (Portland, OR, USA) and cultured by provider's the recommended protocol.

Primary tumor tissues

Eleven cases of cervical SCCs, in the form of formalin fixed, paraffin embedded, archival tissue, were obtained under the Institutional Review Board approved protocol (CUMC10U917) at The Catholic University of Korea, College of Medicine.

Real-time quantitation of copy number

Genomic DNAs were extracted from cultured cells using the QIAmp DNA mini kit (Qiagen, Valencia, CA, USA). SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) was used to amplify target (*BCAS1*) and reference (beta actin, *ACTB*) genes using a LightCycler 480 II (Roche, Rotkreuz, Switzerland). The following oligonucleotides were used as primers: *BCAS1*-F 5'-GGTGTCTCTGTGGAGAGGTGT-3'; *BCAS1*-R 5'-ACTA-AGGTGCAGAGGCCTGA-3'; *ACTB*-F 5'-AGAAAATCTGGCACCACACC-3'; *ACTB*-R 5'-AACGGCAGAAGAGAGAACCA-3'. After pre-incubation at 95°C for 5 minutes, 45 cycles of amplification reaction (95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) were performed. After amplification, melting curves were analyzed to confirm specific amplification reactions; all samples were performed in triplicate. Pooled genomic DNA of human lymphocytes (Promega, Madison, WI, USA) was used as a calibrator. We used the E-method of LightCycler 480 II (Roche) to calculate template concentration; the copy number of the target and reference were normalized by the calibrator.

Real-time quantitation of mRNA

Total RNAs were isolated from cultured cells using RNAiso Plus (Takara Bio Inc.) using the manufacturer's recommended protocol. One Step SYBR PrimeScript RT-PCR kit II (Takara Bio Inc.) was used to synthesize cDNA and amplify target (*BCAS1*) and reference (alpha tubulin, *TUBA1B*) sequences in a single step using a LightCycler 480 II (Roche). The following oligonucleotides were used as primers: *BCAS1*-F 5'-CAAGCAGGAAGCCAAAGAAC-3'; *BCAS1*-R 5'-GGAACCGTGCTGATTGTGTTT-3'; *TUBA1B*-F 5'-TGGAACCCACAGTCATTGATGA-3'; *TUBA1B*-R 5'-TGATCTCCTTGCCAATGGTGTA-3'.²³ After cDNA synthesis at 42°C for 5 minutes, 45 cycles of amplification reaction (95°C for 5 seconds, 60°C for 10 seconds, and 72°C for 10 seconds) were performed. After amplification, melting curves were analyzed to confirm specific amplification reactions; all samples were performed in triplicate. We used the same method for the quantification of copy number to calculate the normalized mRNA ratios.

Electrophoresis and immunoblotting

After washing the cultured cells with ice-cold 1× phosphate buffered saline, the cells were harvested by scraping into ice-

cold lysis buffer (150 mM sodium chloride, 1.0% NP-40, 50 mM Tris, pH 8.0) with 0.5 mL of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Cells were agitated at 4°C for 30 minutes and centrifuged at the speed of 12,000 rpm for 20 minutes. The supernatants were collected for protein quantitation. Next, 10 µg of each protein samples were resolved on sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking the membrane with 5% bovine serum albumin in 1×TBST, the membrane was incubated with 1:2,500 dilution of mouse anti-human *BCAS1* monoclonal antibody (Abnova, Taipei City, Taiwan) at 4°C overnight. The membrane was washed three times with 1×TBST for 5 minutes and incubated with biotin-conjugated, secondary antibody (1:10,000) for 1 hour at room temperature. After incubation with ExtrAvidin-Peroxidase (1:2,000, Sigma-Aldrich) for 30 minutes, the membrane was developed using the Western Lightning Plus ECL (PerkinELmer, Waltham, MA, USA).

RESULTS

Copy number alterations of *BCAS1* gene

Contrary to HEK293, which showed two normal copies of *BCAS1*, 7 (88%) of 8 SCC cell lines tested showed an increased copy number of *BCAS1* at the various degrees (Fig. 1). HCC-1588 did not show a copy number gain of *BCAS1*. FaDu showed

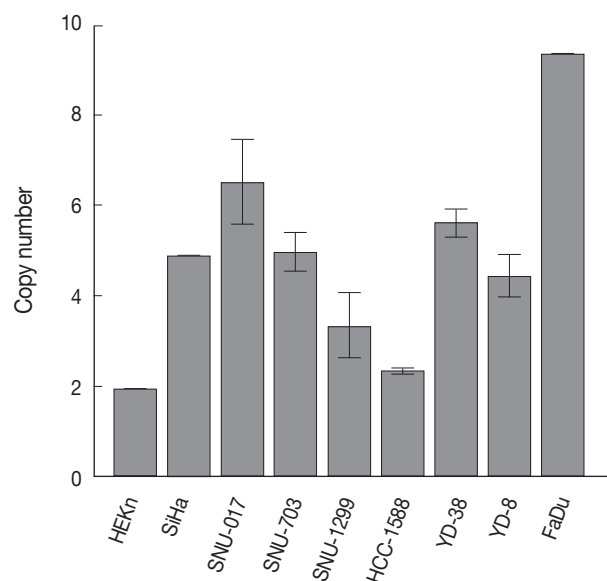


Fig. 1. Copy number alterations of breast carcinoma amplified sequence 1 (*BCAS1*) gene found in squamous cell carcinoma cell lines.

the highest copy number gain for *BCAS1* among the cell lines tested.

BCAS1 expression at RNA level

Even if most of SCC cell lines tested showed an increase in the copy number gain in *BCAS1*, some of them failed to show increase in mRNA expression (Fig. 2). SiHa, HCC-1588 and YD-8 showed lower level of mRNA expression of *BCAS1* than HEK293.

BCAS1 expression at protein level

SiHa, HCC-1588, SNU-1299, and FaDu showed an increase in *BCAS1* protein expression. YD-8, YD-38, SNU-703, and SNU-017 did not show a significant difference in *BCAS1* expression for HEK293 (Fig. 3).

Copy number alterations of *BCAS1* in primary tumors

Among the 11 cases of cervical SCCs, 8 (73%) showed an increase in the copy number of *BCAS1* gene (Fig. 4).

DISCUSSION

We have a screened copy number of *BCAS1* and basal level of

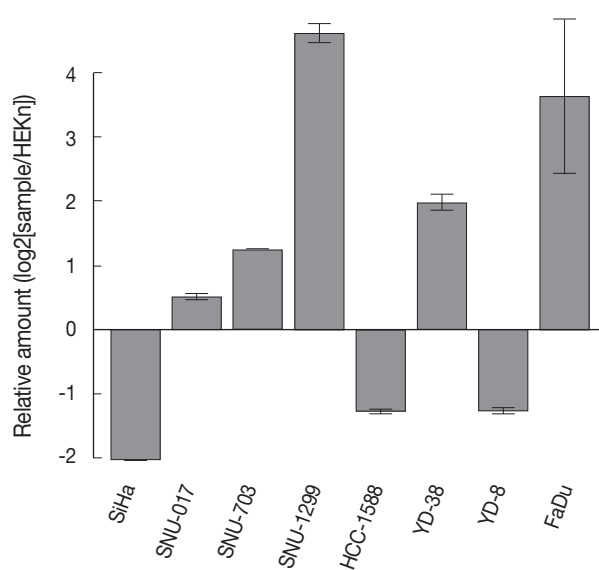


Fig. 2. Quantitation of breast carcinoma amplified sequence 1 (*BCAS1*) mRNA. Some of the squamous cell carcinoma cell lines show increased expression of *BCAS1* mRNA.

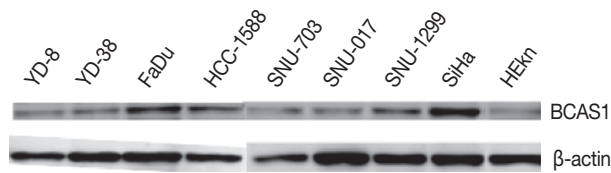


Fig. 3. Immunoblot of breast carcinoma amplified sequence 1 (BCAS1) protein. FaDu, HCC-1588, SNU-1299, and SiHa show an increase in BCAS1 protein. Normal keratinocytes (HEK293) show minimal expression of BCAS1.

its expression in terms of mRNA and protein in the SCC cell lines. We also confirmed copy number gains for *BCAS1* in primary tumors. Most of the SCC cell lines and primary tumors tested in this study showed an increase in the copy number of *BCAS1* for breast cancers. However, these copy number gains were not accompanied by a proportionate increase in *BCAS1* expression. Considering the complex gene interactions in living cells, it is possible to speculate that the effect of the other genes related to *BCAS1* may contribute to the discrepancy between gene dosage and expression level of *BCAS1* as well as between the mRNA and protein levels. Although we could not prove that the overexpression of *BCAS1* is a common finding in SCC cell lines, the fact that the cell lines have frequent copy number gains for *BCAS1*, with variable expression levels, is noteworthy.

Only 40 to 60% of copy number alterations cause changes in gene expression. This is why it is hard to explain so many copy number alterations accompanied by human cancers. A significant portion of the copy number alterations might be caused by other alterations specific to the cell lines or primary tumors. So any genetic or genomic alterations found in cell lines might be caused by random changes. However, the copy number gains of *BCAS1* found in this study show a high frequency (88%) and the alterations are also found in most of the primary cervical SCC tissues tested (73%). This suggests that the copy number gains of *BCAS1* in cell lines do not occur by chance alone. Although this cannot explain the causal relationship between *BCAS1* amplification and SCC carcinogenesis, it is enough to say that the amplification of *BCAS1* is a common finding in SCCs. Furthermore, the cell lines harboring *BCAS1* amplification at the variable expression levels will provide an *in vitro* model to understand the role of *BCAS1* in the carcinogenesis of SCC; further study is required to clarify the function of *BCAS1*.

Carcinogenesis is a multi-step process. To get the end stage of carcinogenesis, several critical genes must be affected. *BCAS1*, which we focus on in this study, might have a significant impact on well-known tumor-related genes or pathways and provide a new facet of their carcinogenesis model. Until now little

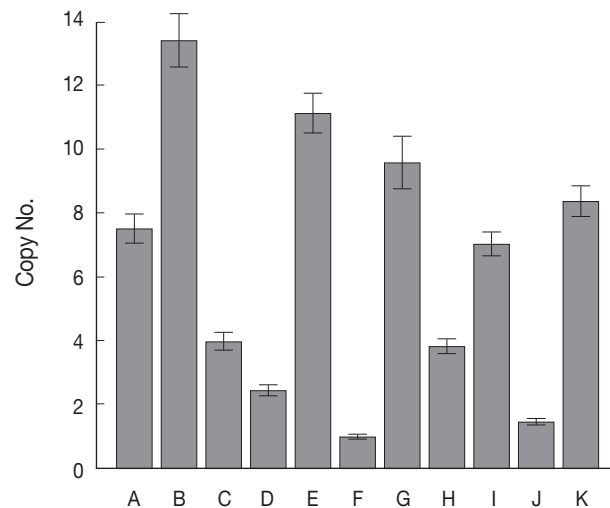


Fig. 4. Quantitation of the breast carcinoma amplified sequence 1 (*BCAS1*) gene in primary cervical squamous cell carcinoma cells shows that 8 out of 11 cases show increased copy number of *BCAS1* gene.

known about the role of *BCAS1* in carcinogenesis. As an effort to understand *BCAS1* in the context of multi-step process, this study will add SCC to the list of malignant tumors in which copy number gain of *BCAS1* may have critical role in its carcinogenesis.

In summary, we showed that copy number gain of the *BCAS1* gene is a common finding in SCC cell lines and primary tumors. These cell lines may be used as an *in vitro* model to delineate *BCAS1* function in tumorigenesis. However, as seen in this study, increased gene dosage does not necessarily mean increased gene expression. To explain the relationship between gene dosage and gene expression of *BCAS1*, further study is required.

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