



Evaluating Expression of the *STAG1* Gene as a Potential Breast Cancer Biomarker

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A B S T R A C T

STAG proteins, which are part of the cohesin complex and encoded by the *STAG* genes, are known as Irr1/Scc3 in yeast and as SA/STAG/stromalin in mammals. There are more variants as there are alternate splice sites, maybe three open reading frames (ORFs) code for three main proteins, including: SA1 (STAG1), SA2 (STAG2) and SA3 (STAG3). The cohesin protein complex has various essential roles in eukaryotic cell biology. This study compared the expression of the *STAG1* gene in four different breast cancer cell lines, including: MCF-7, T-47D, MDA-MB-468, and MDA-MB-231 and normal breast tissue. RNA was extracted from these cell lines and mRNA was converted to cDNA, and then expression of the *STAG1* gene was quantified by three sets of specific primer pairs using Real Time-quantitative PCR (RT-qPCR). The findings show significantly different over-expression of *STAG1* in these cancer cell lines in comparison with the normal tissue, and the cell lines were different in their expression levels. In conclusion, the *STAG1* gene can be postulated as a candidate breast cancer biomarker that needs to be further evaluated in breast tumor biopsies.

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INTRODUCTION

Tumorogenesis is a complex multi-stage biological process caused by many etiological factors (1). There exist numerous cancer biomarkers including: glycomic, proteomic, imaging, epigenetic, and genetic biomarkers, which have been exploited successfully for cancer diagnosis, prognosis and epidemiology. However, few biomarkers with high specificity and sensitivity are available for cancer discovery (2).

One possible group of cancer biomarkers are members of the cohesin complex. Cohesin is a structure of a ring-shape that is composed of four main protein subunits, including: SMC subunits (structural maintenance of chromosome), these in turn, include Smc1 and Smc3, and two non-SMC protein subunits include: Scc1/Rad21/Mcd1, and lastly stromal antigen (SA)/Stromalin/Scc3/STAG (3) (Figure 1). The STAG proteins are subunits of the HEAT-repeat domain and take part in protein-protein interactions. In vertebrates, three protein variants have

been recognized including: SA1 (STAG1), SA2 (STAG2) and SA3 (STAG3) (4). In all organisms where STAG/Scs3/Stromalin proteins exist, they are located in the nucleus as well as in the intracellular stroma (5).

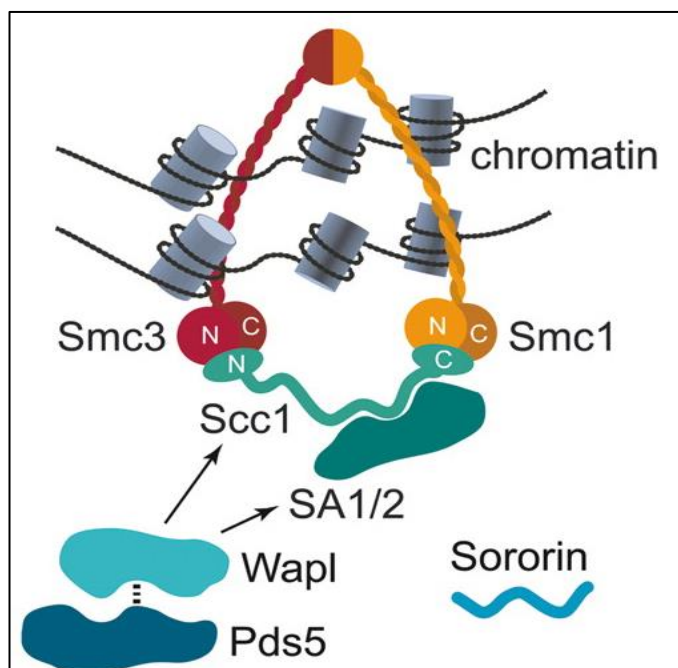


Figure1. The cohesin complex architecture model encircling the chromatin (6)

In mammalian cells, *Drosophila*, fission yeast, and *Caenorhabditis elegans* cohesion interacts with chromosomes during telophase before creation of cohesion in S-phase of the cell cycle. Whereas cohesion association with chromosomes occurs in late G1 phase in budding yeast (7, 8). During the cell cycle, the cohesin complex associates with chromosomes to ensure that pairing of sister chromatids produced during DNA-synthesis persists post-replication until anaphase. This pairing, in turn, allows spindles to recognize mitotic replication products, and later permits bipolar alignment of these mitotic replication products on the mitotic spindles (9).

Importantly, the cohesin protein complex have various critical roles in eukaryotic cell biology, such as cohesion of sister chromatid in mitosis and meiosis, biorientation of chromosomes onto mitotic and meiotic spindles, repair of DNA double strand breaks via homologous recombination, chromosome segregation, cell cycle-checkpoint control, as well as chromatin remodeling (10, 11). Another role for cohesin has been observed through regulating gene expression (12). In each of humans, mouse, *Drosophila*, and Zebra fish, cohesin binds *myc* gene to facilitate its expression, and its protein (Myc) has an important role in the control of cell proliferation and protein production (13). In human cells, it has been found that reduction of cohesin results in abnormal cellular responses to estrogen (steroid) hormone, indicating that cohesin regulates steroid signaling (14). Similarly, knockdown of cohesin reduces the

myc gene expression (15). Cohesin defects are proposed to cause various malignancies (16) and cohesin expression has been found to be dysregulated in many tumours involving breast, prostate, and oral squamous cell carcinoma; furthermore, mutations in genes encoding cohesin components have been reported in colorectal and myeloid malignancies (17).

Breast cancer biomarkers are needed for detecting both human breast tumors and also for tumors of animal udders. Especially, bitches were reported to undergo several types of mammary tumors that involve multiple glands (18). Recently, the cohesin component STAG3 has been characterized as a potential new biomarker for breast cancer (19). The current study sheds light on the *STAG1* gene, in which its expression was studied in four different human breast cancer cell lines relative to its expression in normal breast tissue. The purpose of the experiment was to discover if there is abnormal expression or not in these breast cancer cell lines, as a prelude to further study of more breast tumours.

MATERIALS AND METHODS

Mammalian Cell Lines

The breast cancer cell lines applied in this study included: MCF-7, T-47D, MDA-MB-231, and MDA-MB-468. These cell lines were obtained from Department of Oncology and Metabolism, Medical school, The University of Sheffield, Sheffield, UK. In addition, human normal total RNA of breast was used as a control (BioChain Institute, Inc., Lot no. B401325).

Primers

Primers for the *STAG1* gene were designed manually for N-terminus, central domain (termed M) and C-terminus of the gene. Either the forward or reverse primer was designed at the junction separated the two exons in order to prevent possible amplification of genomic DNA. Furthermore, the primers were chosen to amplify amplicons of approximately 100 nucleotides. β -Actin was used as endogenous reference gene. Testis cDNA was used as a positive control to check the amplification of *STAG1* by these primers before examining cDNA of samples. Primers are shown in the Table below.

Table . Primer pair sets used in this study (20)

Oligomer	Symbol	5'-3' sequence
STAG1 For N	STAG1 N	AAAGAAAAAGGGGTGCTCCTGGCCG
STAG1 Rev N		CCTGCTCCTCTAATTCAGCTTC
STAG1 For M	STAG1 M	CATGGAGCCATGCCAGAACAG
STAG1 Rev M		CCTCTTTGGAAGGAGAGCCATC
STAG1 For C	STAG1 C	GTGATGATGTCATCCCAAGCC
STAG1 Rev C		CAGCTCTCTCGCCGATTTC
β -Actin For	β -Actin	CAGCCATGTACGTTGCTATCCAGG
β -Actin Rev		AGTCCAGACGCAGGATGGCATG

Cell Culture Medium

The culture medium used to grow the breast cell lines was Dulbecco's modified eagles medium (DMEM; Lonza; 500 mL). In addition, fetal calf serum (FCS; prepare 10%), non-essential amino acids 100x (NEAA; prepare 1%), with or without antibiotics (1% of 1x Penicillin/ Streptomycin were also added. Before use, the culture medium, Trypsin/Versin (Lonza), and phosphate buffered saline (PBS) were warmed in a 37 °C water bath for approximately 30 min.

Freezing Medium

The freezing medium used to store breast cancer cell lines for long term was made by mixing 10% Dimethyl Sulphoxide (DMSO) with the complete DMEM medium described above.

Cell Maintenance

Using the above cell culture medium with or without antibiotic was used for growing the breast cancer cell lines in T75 (160 mL) flasks. Then, the cultivated flasks were incubated horizontally at 37 °C and 5% CO₂ in a humid incubator. The cells were inspected under the light microscope to examine cell confluence nearly every day or every three days based on the cell line used.

Cell Passage

Confluent cells were split as necessary every 3 days or sometimes more based on the cell type. The medium was removed from the flask, followed by washing with 10 mL PBS. After removal of PBS, 1 mL of Trypsin/Versin was added over the cells, which were left in an incubator at 37 °C and 5% CO₂ for 1-2 min or until detachment of the cells from the flask. Then, the trypsin effect on the cells was inhibited by adding 9 mL of fresh cell culture medium, which was mixed vigorously with the trypsinized cells about 10 times by up and down pipetting. Finally, the cell growth was maintained by dispersing these 10 mL into other flasks as required.

Harvesting

Cells were grown to about 80% confluence and harvested as mentioned above to obtain 10 mL of the medium mixed with trypsinized cells. Cells were spun at about 1500 rpm for 3 min. Afterwards, the cell pellets were collected and either mixed with 1 mL freezing medium into a cryogenic vial (Corning) and stored for long term in -80 °C liquid nitrogen. Otherwise, the cell pellets were washed twice with PBS at 1500 rpm for 3 min each, and then re-suspended in 1 mL PBS in an Eppendorf tube and centrifuged as above. Lastly, the PBS was discarded, and the pellets stored at -20 °C for RNA extraction.

Thawing

To thaw the frozen cells, they were warmed at 37 °C in a water bath for ~ 1 min. Then, the cells were mixed with 9 mL of pre-warmed cell culture medium into a T25 (40 mL) flask. These flasks containing cells were incubated horizontally at 37 °C and 5% CO₂ for 24 h before moving them to T75 flask.

Cell Viability and Counting

The cell viability was checked by using 0.4% (w/v) Trypan blue stain (Sigma-Aldrich). The cell suspension was mixed with the stain in 1:1 ratio inside an Eppendorf tube with shaking, then a suitable amount was added into hemocytometer. The viable cells looked un-stained under the light microscope. Under the microscope, the cell numbers were counted in the four squares of the hemocytometer and multiplied by 2. The resulting number was multiplied by $\times 10^4$ cells/mL.

RNA Extraction

Whole cell RNA was extracted from the cell pellets by using RNeasy® Mini Kit (Qiagen) following the Manufacturer's instructions (Quick-Start Protocol). Then, the RNA concentration was measured as described below, and kept at -80 °C for long term storage.

Conversion of RNA to cDNA

Following the manufacturer's instructions, RNA was extracted and converted to cDNA using a High-Capacity RNA-to-cDNA kit (AB Applied Biosystems). The cDNA was stored in -80 °C till use. Serial dilutions of 1/5, 1/25, 1/125 and 1/625 were made from each breast cancer cell type cDNA sample. In addition, a dilution of 1/50 was prepared from some cancer cell lines to choose the best dilution to be used later to study the expression of the *STAG1* gene relative to β -Actin (the reference gene).

Calculating Nucleic Acid Concentration

The concentrations of RNA and DNA were measured using NanoDrop™1000 spectrophotometer (Thermo Scientific).

Real Time-quantitative PCR (RT-qPCR)

In this study, RT-qPCR was run by using a Corbett Robotics Rotor-Gene™ 6000 (Qiagen). The reaction components consisted of 10 μ L of 2 \times SensiMix (containing a mixture of dNTP, buffer, HiRox, SYBR Green, and Taq polymerase), 3 μ L sterile distilled water, and 2 μ L of 10 \times primers were used with 5 μ L cDNA template. The final volume of the reaction was 20 μ L. No template controls

were used in the whole experiment. All reactions were carried out in triplicate for the normal tissue and cell lines.

Melt curve analysis was used to make sure there was no primer dimer with the amplification of a single product. The PCR cycling conditions included 10 min for 95 °C for activation of Taq polymerase, followed by 40 cycles of denaturation for 15 sec at 95 °C, annealing for 15 sec at 58 °C and lastly extension for 30 sec at 72 °C. The quantity of the PCR product was proportional to the fluorescence signal. By using software specific for the Rotor-Gene 6000, the C_T (the threshold) value was estimated for the cDNA samples in each reaction.

Preliminary RT-qPCR test using manual serial cDNA dilutions (1/5, 1/25, 1/125, and 1/625) from each sample of breast cancer cell line was carried out with β -Actin and STAG1 primers to choose the best dilution in order to use it afterwards in the following trials. Then, serial ten-fold dilutions (1/10, 1/100, 1/1000 and 1/10000) were made from cDNA of some breast cancer cells in order to evaluate whether the amplicons of the reference and target genes have nearly the same amplification efficiency. These cDNA dilutions were amplified by using qPCR targeting gene-specific primers. The ΔC_T values were calculated for the dilutions, and the amplification efficiency was determined using the formula $E = 10^{(-1/\text{slope})}$ (21).

According to (22), the value of relative gene-expression was calculated using the formula: $\Delta C_T = C_T \text{ target gene} - C_T \text{ endogenous reference gene}$, admitting for samples' comparison independently of the total cDNA input amount. As the average amplification efficiency was 1.9; therefore, the parameter $1.9^{-\Delta C_T}$ was used for comparing the expression levels among genes in the cell line itself. This represents the expression fold of one gene with respect to a reference gene.

Statistical Analysis

Sample expression results are shown as the median and range rather than the mean. Nonparametric, Mann Whitney U test, was used for results analysis, in which differences of expression level between a sample of breast cancer cell line and another of normal breast were considered significant ($P \leq 0.05$) when confidence levels were larger than 95%.

RESULTS

Preliminary RT-qPCR Tests

Among the different dilutions of cDNA, the dilution 1/50 showed results of amplification better than all of the other primers. Therefore, this dilution was depended throughout all qPCR reactions. Figure 2 demonstrates how the amplification efficiency of each target, and the reference gene was calculated depending on the variations of ΔC_T . The ΔC_T varied with template dilution, and it averaged 1.9 as mentioned above.

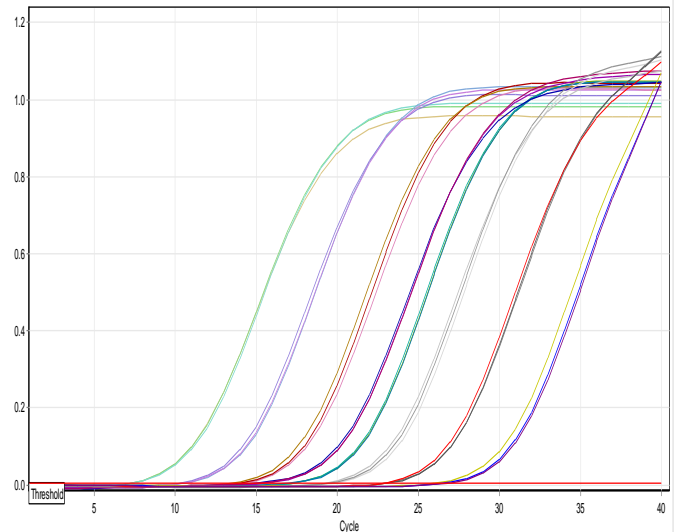


Figure 2. RT-qPCR preliminary test shows the amplification curves, represented by C_T values, of the target and reference genes in ten-fold serial dilutions of the breast cell lines' cDNA

RT-qPCR Amplification Experiments

The three primers bound at different regions of *STAG1* successfully amplified it using RT-qPCR, with C_T values ranging from 16 to 32. For each RT-qPCR reaction, clean melt curves were observed without any primer dimers as shown in Figure 3. Box and whiskers (Figure 4) shows the expression levels of the targeted gene relative to β -actin in breast normal and cancer cell lines.

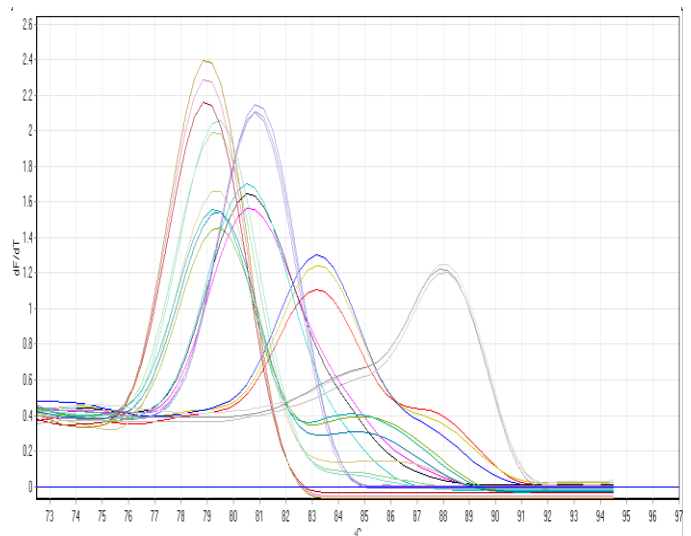


Figure 3. The melt curve of RT-qPCR reaction shows no primer dimers, but only *STAG1* amplified by the three sets of primer pairs and β -actin amplification in one of breast cancer cells' cDNA is shown

The Amplification Potency of the Three Primers Designed for the Same Gene Was Different

The three sets of primer pairs gave various amplification levels in RT-qPCR. Interestingly, *STAG1* M showed significantly higher levels in the studied cancer cell lines, excluding T-47D cells where no significant difference was reported relative to normal breast tissue (Figure 4).

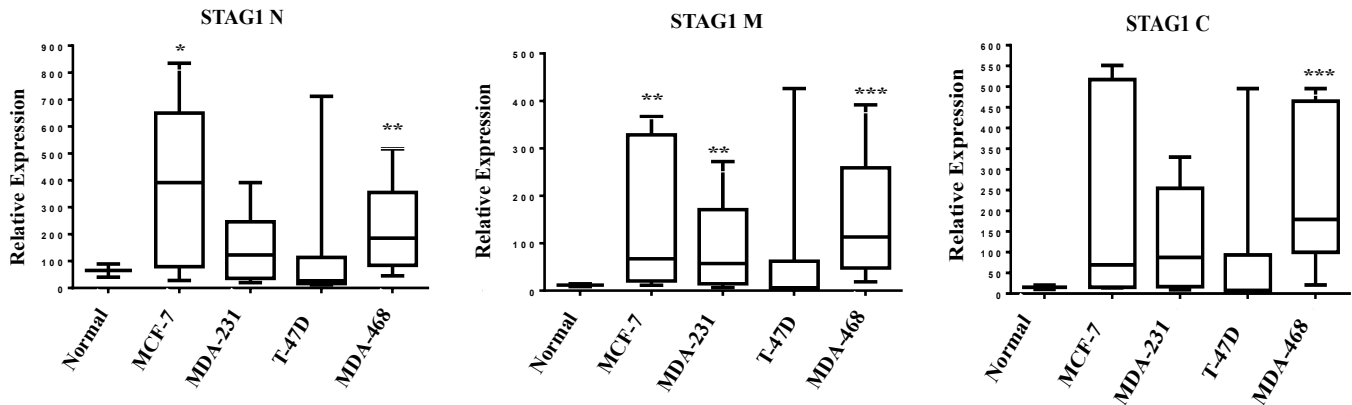


Figure 4. Expression of the *STAG1* gene in normal breast cDNA sample and cDNAs from breast cancer cell lines, with the horizontal lines represent median. Three primer pairs (STAG1 N, STAG1 M and STAG1 C) with β -Actin as an internal reference gene were used. Expression levels were plotted on a linear scale to allow comparison of high and low levels

Gene Expression Was Different Among the Breast Cancer Cell Lines

Three of the studied cancer cell lines showed *STAG1* up-regulation as compared to the normal breast tissues. MCF-7 showed marked overexpression of the target gene. Moreover, MDA-MB-468 cells were up-regulated more strongly than MDA-MB-231.

The Relationship Between the Tumor Type of Cancer Cell Line and Expression Level

To determine whether *STAG1* is expressed or not, the luminal cancer cell lines, MCF-7 and T-47D, in addition to MDA-MB-468 and MDA-MB-231, which are the basal A and B cell lines, respectively were compared. Interestingly, the expression in the luminal cancer cells (MCF-7 and T-47D) was markedly different despite the fact that both cell lines have some important mutual characteristics. Noticeably, T-47D cells revealed rather different expression compared to MCF-7.

Concerning basal cells MDA-MB-231 and MDA-MB-468, the last one was clearly much over-expressed for *STAG1* compared to the first despite both are sharing many features. These findings demonstrate that breast cancer cell type might be the cause behind the difference of gene expression reported in this research.

DISCUSSION

Genes encoding cohesins and their regulatory and associated proteins might be genome stability genes. This makes sense as cohesin is vital to controlling chromosome segregation, and missegregation of chromosome can in turn be a source of loss of heterozygosity. Whether cohesin effects occur in cancer initiation, progression or both is unascertained as is which cohesin components occur in various physiological and pathological situations (16).

This study focused on breast cancer as it is the main cause leading to death in women all over the world. Furthermore, it is a disease of molecular heterogeneity that possesses many markers, such as, ER (estrogen receptor), PR (progesterone receptor), and ERBB2/HER2 (human epidermal growth factor receptor 2). These markers have been exploited to classify cancer patients according to suitability of targeted treatments, as well as the markers were used for cancer prediction (23).

The RT-qPCR assay was used in the current study to quantify the *STAG1* gene expression in different breast cancer lines. This approach has been verified as the best alternative to cDNA microarrays to profile molecular tumor (24). Furthermore, RT-qPCR is easy to perform, highly sensitive that permits quantification of rare mRNA levels and small alterations in gene expression, and cost effective compared to RNA sequencing for single gene analysis (21).

Interestingly, Croucher (25) reported under-expression of *STAG1* in breast tumors as compared to normal breast tissues. These observations concerning breast tumors are consistent with those of (24) who found the *STAG1* gene significantly down-regulated in invasive breast tumors. However, the findings here revealed marked up-regulation of *STAG1* in three of the studied cancer cell types (MDA-MB-468, MDA-MB-231 and MCF-7) relative to normal breast. Importantly, the primers designed for binding with the central region of *STAG1* were better in terms of gene amplification than the other primers. Overexpression of cohesin in cancers advances the possibility that cohesin contribute to cancer progression via transcription regulation in two ways: firstly, by regulation of pluripotency genes and oncogenes; secondly, by its influence on hormone-dependent pathways motivating cancer (17). By contrast to other cell lines, T-47D did not reveal a significant difference in *STAG1* expression compared to normal tissue. This conflict in expression between breast tumors and cancer cell lines has been explained by Kao and his co-workers (26) by reporting that

at the genomic level it is unclear how well cell line subtypes truly represent their counterparts of tumor subtypes, i.e., the cancer cell lines neither be completely compatible nor respond the same as tumorous tissues.

It is remarkable in this study to see the breast cancer cell lines differ in their *STAG1* expression compared to the normal breast tissue. High expression of this gene was exerted by the MCF-7 cells by most of the primers. In contrast, the expression in T-47D cells was much lower than the other cancer cell lines despite the fact that both T-47D and MCF-7 cells have some mutual characteristics; both are luminal, ER and PR positive, and HER2 negative. The only difference between these cell lines is that the tumor type of MCF-7 cells is metastatic adenocarcinoma, while the T-47D type is invasive ductal carcinoma (26). Noticeably, the later cell line showed completely different gene amplification by the primer sets used.

Concerning basal cells (MDA-MB-231 and MDA-MB-468) used in the current study, their *STAG1* expression levels were higher than the normal breast tissue. However, the second one was noticeably much over-expressed compared to the first one. Although, both share common characteristics of being triple negative (ER, PR and HER2 negative) and their tumor type is metastatic adenocarcinoma. While MDA-MB-468 is basal A subtype, MDA-MB-231 is basal B subtype (26). Our findings showed significant over-expression for MDA-MB-468 in almost all of the primers used, consequently, it might be a good alternative to MCF-7, as MCF-7 cell lines have been considered the standard workhorses that many researchers prefer to work with in their studies rather than thinking of different breast cancer lines.

In conclusion, the *STAG1* gene can be postulated as a candidate breast cancer biomarker that needs to be further evaluated in numerous breast tumor biopsies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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تقييم التعبير الجيني لجين *STAG1* كمؤشر بيولوجي محتمل لسرطان الثدي

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الخلاصة

تُعرف بروتينات STAG، التي تعد جزءًا من معقد cohesin والمشفرة بواسطة جينات STAG، باسم Irr1/Scs3 في الخميرة و SA / STAG / stromalin في الثدييات. جرى التعرف على ثلاثة أنواع رئيسية من هذا البروتين في الفقريات، بما في ذلك: SA1 (STAG1) و SA2 (STAG2) و SA3 (STAG3). تلعب بروتينات معقد cohesin أدوارًا أساسية مختلفة في بيولوجيا الخلية حقيقية النواة. قارنت هذه الدراسة التعبير الجيني لجين STAG1 في أربعة أنواع مختلفة من خلايا سرطان الثدي، بما في ذلك: MCF-7 و T-47D و MDA-MB-468 و MDA-MB-231 وأنسجة الثدي الطبيعية. جرى استخلاص الحمض النووي الريبوزي RNA من خطوط الخلايا هذه وتم تحويل mRNA إلى cDNA، ثم تم تحديد كمية التعبير عن جين STAG1 بواسطة ثلاث مجموعات من أزواج البواديء باستخدام تفاعل سلسلة البلمرة الكمي أظهرت النتائج اختلافًا كبيرًا في تعبير STAG1 في خطوط الخلايا السرطانية هذه مقارنةً بالأنسجة الطبيعية، وكانت أنواع الخلايا مختلفة في مستويات تعبيرها. في الختام، يمكن افتراض استعمال جين STAG1 كمعلم أو مؤشر بيولوجي للتحري عن سرطان الثدي، وهذا يحتاج إلى مزيد من التقييم في خزعات ورم الثدي.

الكلمات المفتاحية: *STAG1*، التعبير الجيني، سرطان الثدي، المعلم الحيوي