# The Association of Co-expression of CD44v4/MMP-9 with Different Nodal Status in High-Grade Breast Carcinoma Patients

Voranuch Thanakit MD*, Pichet Sampatanukul MD*,
Preecha Ruangvejvorachai*, Somboon Keelawat MD*

* *Department of Pathology, Faculty of Medicine, Chulalongkorn University*

***Background:*** *Breast carcinoma is one of the most common tumors in female patients, and its metastasis is a major cause of death. An experimental model has recently found the association of CD44 with MMP-9 that facilitates tumor cell invasion and metastasis.*
***Material and Method:*** *The CD44v4 and MMP-9 were performed on tissue in paraffin blocks of 50 cases of high-grade breast carcinoma with node positive and 50 cases with node negative.*
***Results:*** *Increased expression of MMP-9(60%) significantly observed in high-grade breast carcinoma patients with node positive (p = 0.004), whereas CD44v4 displays no significant difference between the two groups (p-value = 0.81). Significant co-expression of CD44v4+/MMP-9+ (46%) was observed and correlated with node-positive patients whereas the CD44v4+/MMP-9- (54%) express in node-negative patient (p-value = 0.01).*
***Conclusion:*** *The solely expression of CD44v4 does not associate with node status. MMP-9 plays an important role to enhance breast carcinoma cell invasion and associates with lymph node metastasis. The combined expression of CD44v4 (overexpression) and derangement of MMP-9 expression was significantly associated with nodal status.*

***Keywords:*** *Breast carcinoma, CD44v4, MMP-9, Lymph node status*

***J Med Assoc Thai 2005; 88(Suppl.4): S30-5***
***Full text. e-Journal:*** *http://www.medassocthai.org/journal*

Breast carcinoma is one of the most common tumors in women, and its metastasis is a major cause of death. The status of axillary lymph nodes is the most important major prognostic factors for invasive breast carcinoma in non-distant metastasis groups[1]. The 5-year survival rate of these two groups is contrasting, and the node-negative group has more favorable outcome.

The understanding of phases and detail of tumor invasion and metastasis is in progress. Each step of this process has a great number of protein assembly to encourage tumor cells to interact with their extracellular matrix and surrounding microenvironment to generate a pathway that facilitates tumor cell motility.

*Correspondence to : Thanakit V, Department of Pathology, Faculty of Medicine, Chulalongkorn University, Rama IV Rd, Bangkok 10330, Thailand. Phone: 0-2256-4235, 0-9022-2112, E-mail: voranewt@yahoo.com*

We here focus on the biological activity of the tumor cells that is essential for invasion and metastasis. The adhesion molecule and the regulation of proteolysis are assessment as the crucial role of invasiveness and metastasis. Although several adhesion molecules and matrix metalloproteinase have been shown to function in the part of metastasis, several evidences from *in vitro* studies on mouse mammary carcinoma cells have found a coordinated function of the tumor cells associated with adhesive and proteolytic acitivity and indicated CD44-MMP-9 has a role in regulating tumor cell invasiveness. The CD44-associated tumor invasion and promote tumor invasion via the regulation of protease activity to digest basement membrane and extracellular matrix. CD44 has been shown to anchor MMP-9 on the cell surfaces and facilitates the tumor cell invasion and dissemination[2]. The disruption or breaking up of this

mechanism may play an important role in the inhibition of the spread of the tumor. Additionally, this is the first investigation into the association of combined CD44v4/MMP-9 expression with different nodal status in clinical material. If there is any correlation, this will provide a useful information to explore in the further study.

CD44 is one of the cell adhesion molecules that implicates the development of malignant tumor and its progression. Its function is lymph node homing, cell-matrix adhesion, and T-lymphocyte activation. The other essential function is transmembrane link molecule for hyaluronan within the extracellular matrix. CD44 molecule is divided into three parts: extracellular domain, transmembrane domain, and the intracellular tail. Its gene is composed of at least 20 exons, the standard form of CD44 (CD44s) and CD44variant isoforms. The numerous isoforms derived from exons 6-15, so-called v1 to v10. The correlation of the variant isoforms expression within different type of tumors has been demonstrated. However, breast carcinoma revealed variable staining of CD44 isoforms. The derangement of CD44 isoforms was analyzed, and its heterogeneity was found. It was established that the progressive loss of CD44v3 and V6 and slight increase CD44s and CD44v5 in less differentiated tumor. In contrast with CD44v4 expression that significantly gain in high-grade (grade3) invasive ductal carcinoma and seems to promote the metastasis of the tumor[3-7]. The CD44v4 appears to be essential for tumor invasion and metastatic spread from previously published data[4].

Another important step of tumor invasion requires its capacity to degrade or breakdown the basement membrane, extracellular matrix and surrounding tissue. Of this, the most important proteolytic enzyme for connective tissue dissolution is metalloproteinases (MMPs)[8,9]. MMPs are divided into subgroups, such as collagenases, gelatinases, stromelysins, and membrane-type MMPs. The matrix metalloproteinase 9 (MMP-9) is a member of the family of metallo-proteinase, also known as gelatinase B or 92-kd Type IV collagenase[4]. MMP-9 performs an influence role to enhance tumor invasion via degradation of the basement membrane and extracellular matrix, angiogenesis, and metastasis. This enzyme is secreted as a stable, inactive zymogen, as proenzymes (pro-MMP-9), and subsequently activated by the cleavage of the N-terminal propeptide for catalytic function[10]. The recent studies have demonstrated conflicting data regarding the association of MMP-9 expression with lymph node status. Some reports indicate the expression of MMP-9 is related to lymphatic metastasis[11,12].

Although, the incidence of axillary lymph node metastasis is not dependent on the grade of the tumor, but a previously report designated a variable expression of both CD44v4 and MMP-9 with grading and found the highest expression in poorly differentiated or high-grade tumor[4,7]. We decided to study in the frame of high-grade invasive ductal carcinoma, NOS with different lymph node status, so the grading of the tumor does not influence the expression of both proteins.

The purpose of this research is to investigate the combined various expressions of CD44v4/MMP-9 and individually expression of CD44v4, MMP-9 associated with high-grade breast carcinoma patients with positive and negative axillary lymph nodes.

## Material and Method

### *Tumor samples*

The tumor samples were collected, with the approval of the Ethics Committee of the Faculty of Medicine, from the modified radical mastectomy specimens, Chulalongkorn University since January 2003 to January 2005. All sections of modified radical mastectomy specimens and submitted lymph nodes were simultaneously reevaluated for histology type, grade, and nodal status. The grading of the tumor is based on the Scarff-Bloom-Richardson system. After the exclusion of ineligible patients and inadequate specimens, 100 patients with high-grade invasive ductal carcinoma were recruited. Of these, 50 individual cases of high-grade invasive ductal carcinoma with node-negative and node-positive were obtained.

### *Immunohistochemistry*

We used 4 -mm sections of paraffin-embedded tissue from the primary tumor. The avidine-streptavidin method was used. The monoclonal antibodies CD44v4 (Novocastra, clone VFF-11) dilution 1:25 and MMP-9 (Neomarker, polyclonal) dilution 1:200 were applied.

All cases were examined by two independent pathologists. Discrepancies between the observers were found in < 10% of the examined slides, and consensus was reached on the further review.

The CD44v4 was considered positive (overexpression) when cytoplasmic or membranous staining more than 10% of tumor cells.

The general expression of MMP-9 in tumor cells was high and the median value from previously report was 85% which was used as a cutoff value for MMP-9 expression in tumor cells[5]. MMP-9 was considered positive staining in tumor cells and was

assessed as semiquantitative. The staining intensity was subjectively scored as weak, moderate, and intense. The moderate and intense staining patterns which cytoplasmic staining > 85% were classified as high expression of MMP-9 (MMP-9+). The others are considered as reduced expression of MMP-9 (MMP-9-).

The co-expression of CD44v4/MMP-9 was divided into 4 groups as follows: CD44v4+/MMP-9+ (Group 1), CD44v4+/MMP-9- (Group 2), CD44v4-/MMP-9+ (Group 3), CD44v4-/MMP-9- (Group4).

***Statistical analysis***

The Chi-square test was used to analyze the relationship between individual expression of CD44V4 and MMP-9 and lymph node status. P value of less than 0.05 was considered statistically significant.

The association between the deranged co-expression of CD44v4/MMP-9 with nodal status was evaluated in three correlative items as follows: Group 1 and 2, 1 and 3, and 1 and 4. Chi-square test was performed and p-value of less than 0.05 was considered statistically significant.

## Results

***The co-expression patterns of CD44/MMP-9 in high-grade breast carcinoma with different lymph node status***

All 100 cases were evaluated and classified into 4 groups. The co-expression in group 1 (CD44v4+/MMP-9+) is definitely classified as positive and detected 23 (46%) in the node-positive group and 12 (24%) in the node-negative group. Group 2 (CD44v4+/MMP-9-) revealed 14 (28%) and 27 (54%) in the node-positive and the node-negative group. Group 3 (CD44v4-/MMP-9+) found 7 (14%) in the node-positive patients and 3(6%) in the node-negative group. Group 4 (CD44v4-/MMP-9-) had 6 (12%) of the node-positive patients and 8(16%) of node-negative group. Of this, the highest expression of both proteins in node-positive patients was overexpression of CD44v4 and high expression of MMP-9 (46%). The others expression patterns of both proteins over the node-positive group including group 2, 3, 4 were 28, 14, and 12%, respectively. In node-negative patients, the predominant pattern was an overexpression of CD44v4, and reduced expression of MMP-9 (54%). The remaining groups (1, 3, 4) found 24, 6, and 16%, respectively (Table 1).

***The relationship of the co-expression pattern of CD44v4/MMP-9 and different lymph node status***

When the co-expressions of both proteins were tested as subgroups based on the different lymph node status. We obtained a significant co-expression of CD44v4+/MMP-9+ in node-positive patients and CD44v4+/MMP-9- in node-negative patients (p-value = 0.01). Regarding other correlated groups, we found no association between group 1 and 3 (CD44v4+/MMP-9+ and CD44-/MMP-9+) and node status (p-value = 0.89). Also, the final related groups did not show any correlation between group 1-4 (CD44v4+/MMP-9+ and CD44v4-/MMP-9-) with different nodal status (p-value = 0.25) (Table2).

***Expression of MMP-9 in high-grade breast carcinoma association with different nodal status***

The expression of MMP-9 was normally positive in cytoplasmic of normal breast tissue, breast cancer cells, stromal fibroblast, and surrounding inflam-

**Table 1.** The co-expression of CD44v4 and MMP-9 and lymph node status

| Co-expression / Nodal status | Group 1 CD44v4+/MMP-9+ case (%) | Group 2 CD44V4+/MMP-9- case (%) | Group 3 CD44V4-/MMP-9+ case (%) | Group 4 CD44v4-/MMP-9- case (%) | Total |
|---|---|---|---|---|---|
| Node positive | 23 (46%) | 14 (28%) | 7 (14%) | 6 (12%) | 50 (100%) |
| Node negative | 12 (24%) | 27 (54%) | 3 (6%) | 8 (16%) | 50 (100%) |

**Table 2.** The relationship of co-expression pattern of CD44v4/MMP-9 with node status

| Group | Correlative item with lymph node status | Chi-square test (p-value) |
|---|---|---|
| 1-2 | CD44v4+/MMP-9+and CD44v4+/MMP-9- | 6.32 (0.01) |
| 1-3 | CD44v4+/MMP-9+ and CD44v4-/MMP-9+ | 0.02 (0.89) |
| 1-4 | CD44v4+/MMP-9+ and CD44v4-/MMP-9- | 1.32 (0.25) |

matory cells[15]. The high expression of MMP-9 in tumor cells was observed in 30/50 (60%) and 15/50 (30%) cases of nodal positive and negative cases, respectively (Fig. 1). The evaluation of MMP-9 with regard to different nodal status group revealed that the high-grade invasive ductal carcinoma showed the significant high expression of MMP-9 in tumor cells of patient within nodal positive group (p-value= 0.004) (Table3).

***Expression of CD44V4 in high-grade breast carcinoma association with different nodal status***

The expression of CD44v4 in 100 cases of high-grade breast carcinoma (both node-positive and node-negative patients) was studied. Among the normal breast epithelial cells, luminal epithelial cells were negative whereas myoepithelial cells and occasionally surrounding stromal cells showed strongly positive staining. The membranous and cytoplasmic staining for CD44v4 in neoplastic cell was identified in 37 of the 50 cases of node-positive patients and 39 of 50 cases of node-negative patients (Fig. 2). The percentage of positive (overexpression) cases for CD44V4 in node-positive and node-negative patients is 74 and 78%, respectively (Table1). Thus, the expression of CD44v4 of these two groups is nearly similar and has no statistical difference (p-value = 0.81) (Table3).

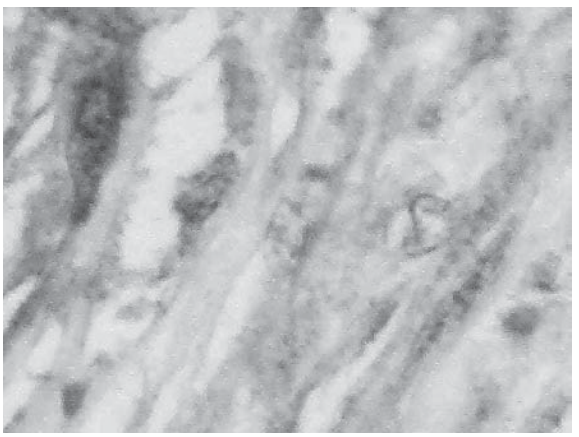

**Fig. 1** High grade breast carcinoma cells exhibits intense staining of MMP-9 within cytoplasm

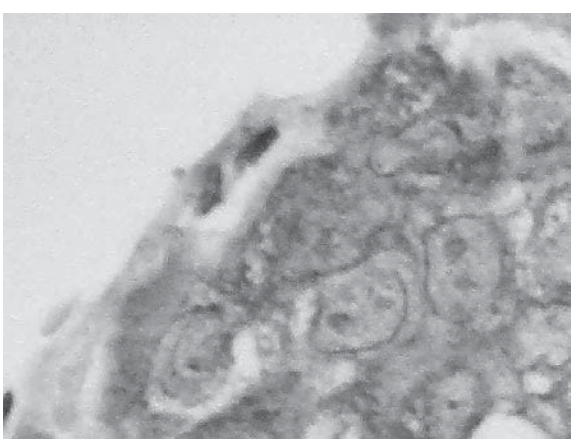

**Fig. 2** The immunohistochemistry study for CD44v4 reveals diffuse cytoplasmic and membranous staining of tumor cells

**Discussion**

Breast carcinoma has a large number of genetic alterations. Some of these provide clues of the treatment, prognostic and predictive values. Until now, the numerous prognostic indicators are still under investigation. Based on this reason, there are continuing searches for the new biological marker of prognosis and more alternative effective roles of the treatment.

In this paper, we provide the immunohistochemical study and analyzed the expression of CD44v4, MMP-9, and co-expression of CD44v4/MMP-9 with various patterns in high-grade breast carcinoma associated with axillary lymph node status. Patients who had node positive demonstrated a significantly high expression of MMP-9 (60%, p-value = 0.004). A number of reports indicated the usefulness of high MMP-9 expression as the indicator for metastatic potential and prognosis of breast carcinoma. The result of this study also corresponds with previous reports, suggesting that MMP-9 plays an influential role in regulating tumor invasiveness and metastasis.

**Table 3.** Relationship of CD44v4 and MMP-9 expression and lymph node status

| Antibody / Nodestatus | CD44v4 (case, %) | | | MMP-9 (case, %) | | |
|---|---|---|---|---|---|---|
| | Positive | Negative | p-value | High | Reduce | p-value |
| Node positive | 37/50 (74%) | 13/50 (26%) | 0.81 | 30/50 (60%) | 20/50 (40%) | 0.004 |
| Node negative | 39/50 (78%) | 11/50 (22%) | | 15/50 (30%) | 35/50 (70%) | |

It has been suggested that an alteration of CD44 isoforms expression implicates the process of tumor movement, invasion and metastasis. A previous study found the different expression of CD44 isoforms in invasive ductal carcinoma. Other protein expressions of CD44 isoforms (v5, v7 and v9-10) do not have any relationship with lymph node status[4,7]. However, the function of CD44v4 is still unclear. In this study, the CD44v4 gains about 76% in high-grade invasive ductal carcinoma, but no significant difference of expression detected between these two groups. The overexpression of CD44v4 in both groups of breast carcinoma patients may suggest that CD44v4 is generated from tumor cells regardless of the nodal status. Thus, the individual expression of CD44v4 does not display any powerful influence on the tumor cells regarding the metastasis.

Interestingly, the combined expression of CD44v4 (overexpression) and high MMP-9 (CD44v4+/MMP-9+) was significantly associated with high-grade breast carcinoma with positive lymph nodes (46%). Whereas, the majority of invasive breast carcinoma with negative lymph node display overexpression of CD44v4, but they reduce the expression of MMP-9 (CD44v4+/MMP-9-), p-value = 0.01. Other patterns of both proteins expression did not show any association with the lymph node status.

A previous *in vitro* study, have linked the expression of CD44 isoforms with MMP-9, and the correlation of CD44 and MMP-9 was found. This was explained by many mechanisms, such as the ectodomain of CD44 splice variants undergo proteolytic cleavage by membrane-associated metalloproteases in breast carcinoma, or CD44 isoforms coprecipitate with MMP-9 without required ligand binding which promotes cell-mediated type IV collagen degradation[13,14]. Our study demonstrates that the overexpression of CD44v4 alone was not directly associated with high-grade breast carcinoma with different lymph node status (p-value = 0.81). However, the relationship between the combined expression of CD44v4 (+) and alteration of MMP-9 expression (+/-) is related to different status of the axillary lymph nodes. We would like to suggest the limited role of the individual CD44v4 associated with lymph node status. Nevertheless, CD44v4 still functions as a part in the step of invasiveness and metastasis.

The node-negative patients also significantly exhibit overexpression of CD44v4 while the MMP-9 was reduced. The individually and co-expression function of MMP-9 also showed significant association with lymph node status. This may support the influential role of MMP-9 to conduct CD44v4 function in tumor cells that action as CD44-mediated tumor which link between the tumor cells and their extracellular matrix. Nevertheless, the overexpression of CD44v4 and high expression of MMP-9 also happen in the node-negative patients (24%). The result of this group may lead us to search for micrometastasis.

In conclusion, the alter expression of protease enzyme play a crucial role to degradation of basement membrane, extracellular matrix, and promote tumor cell invasion and metastasis. Our study found the significant expression of MMP-9 in high-grade breast carcinoma associated with nodal status. However, the CD44v4 was related with MMP-9 as combined expression and mediated tumor cell invasion and metastasis, whereas the solely expression of CD44v4 did not directly associate with lymph node status.

**Acknowledgement**

This work was supported by Ratchadapiseksompotch Fund, grants number RA145/47.

**References**

1. Fitzgibbons PL, Page DL, Weaver D, Thor AD, Allred DC, Clark GM, Ruby SG, O'Malley F, Simpson JF, Connolly JL, Hayes DF, Edge SB, Lichter A, Schnitt SJ. Prognostic factors in breast cancer. College of American pathologists consessus statement. Arch Pathol Lab Med 1999; 124: 966-78.
2. Lee S-C, Harn H-J, Lin T-S, Yeh K-T, Liu Y-C, Tsai C-S, Cheng Y-L. Prognostic significance of CD44v5 expression in human thymic epithelial neoplasms. Ann Thorac Surg. 2003;76:213-8.
3. Rys J, Kruczak A, Lackowska B, Jaszcz-Gruchala A, Brandys A, Stelmach A, Reinfuss M. The role of CD44v3 expression in female breast carcinomas. Pol J Pahol. 2003;54:243-7.
4. Bankfalvi A, Terpe H-H, Breukelmann D, Bier B, Rempe D, Pschadka G, Krech R, Lelle R-J. Immunophenotypic and prognostic analysis of E-cadherin and b-catenin expression during breast carcinogenesis and tumor progression: a comparative study with CD44. Histopathology 1999; 34: 25-34.
5. Berner HS, Suo Z, Risberg B, Villman K, Karlsson MG, Nesland JM. Clinicopathological association of CD44mRNA and protein expression in primary breast carcinomas. Histopathology 2003; 42: 546-54.
6. Friedrichs K, Franke F, Lisboa B-W, Kugler G, Gille I, Terpe H-J, Holzel F, Maass H, Gunthert U. CD44

isoforms correlate with cellular differentiation but not with prognosis in human breast carcinoma. Cancer Research 1995; 55: 5424-33.
7. Foekens JA, Dall P, Klijn JG, Skroch-Anel P, Claassen CJ, Pllk MP, Ponta H, Van Putten W, Herrlich P, Henzen-Logmans S. Prognostic value of CD44 variant expression in primary breast carcinoma. Int J Cancer (Pred Oncol) 1999; 84: 209-15.
8. Eyeblad M, Werb S. New functions for the matrix metaloproteinased in cancer progression. Nat Rev Cancer 2002; 2: 161-74.
9. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001; 17: 463-516.
10. Ramos-DeSimone N, Hahn-Dantona E, Sipley J, Nagase H, French DL, Quigley JP. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enchances tumor cell invasion. J Biol Chem 1999; 274: 13066-76.
11. Iwata H, Kobayashi S, Iwase H, Masaoka A, Fujimoto N, Okada Y. Production of matrix metalloproteinase in human breat cancer. Jpn J Cancer Res 1996; 87: 602-11.
12. Wang LH, Liu DY, Chen YJ, Hou L, Wnag B, Mao LM, Lu S. Relationship between lymph node metastasis and the expression of E-cadherin, N-cadherin, and matrix metallproteinase in breast carcinoma. Ai Zheng 2002: 21: 965-9.
13. Li H, Cao D, Liu Y. Prognostic value of matrix metallproteinases (MMP-2 and MMP-9) in patients with lymph node-negative breast carcinoma. Breast Cancer Res Treat 2004; 88: 75-85.
14. Okamoto I, Tsuiki H, Kenyon LC, Godwin AK, Emlet DR, Holgado-Madruga M, Lanham IS, Joynes CJ, Vo KT, Guha A, Matsumoto M, Ushio Y, Saya H, Wong AJ. Proteolytic cleavage of the CD44 adhesion molecule in multiple human tumors. Am J Pathol 2002; 160: 441-7.

## *ความสัมพันธ์ของการแสดงออกร่วมกันของ CD44v4/ MMP-9 กับสภาวะของต่อมน้ำเหลืองที่แตกต่างกันในผู้ป่วยมะเร็งเต้านมเกรดสูง*

***วรนุช ธนากิจ, พิเชฐ สัมปทานุกุล, ปรีชา เรืองเวชวรชัย, สมบูรณ์ คีลาวัฒน์***

***ความเป็นมา:*** *มะเร็งเต้านมเป็นหนึ่งในมะเร็งที่เกิดขึ้นบ่อยในผู้ป่วยหญิง และการแพร่กระจายเป็นสาเหตุการตายที่สำคัญ จากการทดลองเร็วนี้ๆ พบว่า CD44 มีความสัมพันธ์กับ MMP-9 ซึ่งทำให้เซลล์มะเร็งลุกลามและแพร่กระจาย*
***วิธีการศึกษา:*** *ทำการย้อม CD44v4 และ MMP-9 บนชิ้นเนื้อจากบล็อกพาราฟินของผู้ป่วยมะเร็งเต้านมเกรดสูงที่มีการแพร่กระจายไปสู่ต่อมน้ำเหลือง จำนวน 50 รายและไม่มีการแพร่กระจายไปสู่ต่อมน้ำเหลืองจำนวน 50 ราย*
***ผลการศึกษา:*** *พบค่าการแสดงออกเพิ่มมากขึ้นอย่างมีนัยสำคัญของ MMP-9 (60%) ในกลุ่มมะเร็งเต้านมเกรดสูงที่มีการแพร่กระจายไปสู่ต่อมน้ำเหลือง (p-value = 0.004) ในขณะที่ CD44v4 ไม่พบความแตกต่างอย่างมีนัยสำคัญระหว่างผู้ป่วยทั้ง 2 กลุ่ม (p-value = 0.81) การแสดงออกร่วมกันของ CD44v4+ /MMP-9+ พบ 46% ซึ่งมีความสัมพันธ์อย่างมีนัยสำคัญในผู้ป่วยที่มีการแพร่กระจายไปสู่ต่อมน้ำเหลือง ในขณะที่พบการแสดงออกของ CD44v4+/MMP-9 54% ในกลุ่มผู้ป่วยที่ไม่มีการแพร่กระจายไปต่อมน้ำเหลือง (p-value = 0.01)*
***สรุป:*** *การแสดงออกเดี่ยวๆ ของ CD44v4 ไม่มีความสัมพันธ์กับสภาวะของต่อมน้ำเหลือง MMP-9 มีบทบาทที่สำคัญที่จะส่งเสริมการลุกลามและการแพร่กระจายของเซลมะเร็งไปสู่ต่อมน้ำเหลือง ค่าการแสดงออกร่วมกันของ CD44v4 (เพิ่มมากขึ้น) และการแสดงออกที่แปรเปลี่ยนของ MMP-9 มีความสัมพันธ์อย่างมีนัยสำคัญกับสภาวะของต่อมน้ำเหลือง*