High TNFRSF12A level associated with MMP-9 overexpression is linked to poor prognosis in breast cancer: Gene set enrichment analysis and validation in large-scale cohorts

Jungho Yang¹, Kyueng-Whan Min²*, Dong-Hoon Kim¹*, Byoung Kwan Son³, Kyoung Min Moon⁴, Young Chan Wi⁵, Seong Sik Bang⁶, Young Ha Oh⁷, Sung-Im Do¹, Seoung Wan Chae¹, Sukjoong Oh⁸, Young Hwan Kim⁷, Mi Jung Kwon⁸

¹ Departments of Pathology, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea, ² Department of Pathology, Hanyang University Guri Hospital, Hanyang University College of Medicine, Guri, Gyeonggi-do, Republic of Korea, ³ Department of Internal Medicine, Eulji Hospital, Eulji University School of Medicine, Seoul, Republic of Korea, ⁴ Department of Internal Medicine, Gangneung Asan Hospital, University of Ulsan College of Medicine, Gangneung, Republic of Korea, ⁵ Department of Pathology, Hanyang University College of Medicine, Seoul, Republic of Korea, ⁶ Departments of Internal Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea, ⁷ Departments of Nuclear Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea, ⁸ Department of Pathology, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Anyang, Gyeonggi-do, Republic of Korea

* kyueng@gmail.com(KWM); idavid.kim@samsung.com(DHK)

Abstract

Background
Matrix metalloproteinase-9 (MMP-9) is associated with remodelling of the extracellular matrix and invasion in various cancers. Identifying proteins connected to high MMP-9 expression is important in explaining its mechanisms. Our study aims to shed light on genes associated with high MMP-9 expression and to discuss their clinical impact in breast cancer.

Methods
We evaluated 173 breast cancer cases from the Kangbuk Samsung Hospital, with 1964 cases from the Molecular Taxonomy of Breast Cancer International Consortium database serving as a validation cohort. We investigated relationships between MMP-9 expression and clinicopathological characteristics. We then used gene set enrichment analyses to detect the association of genes with MMP-9 overexpression, and performed survival analyses to determine the significance of the gene in three independent cohorts.

Results
High MMP-9 expression correlated with poor prognosis in univariate and multivariate analyses. Using gene set enrichment analysis, we found that tumour necrosis factor receptor superfamily member 12A (TNFRSF12A) was linked to high MMP-9 expression. In the
survival analysis of three published data sets (METABRIC, GSE1456, GSE20685), high TNFRSF12A was relevant to a poor survival rate.

Conclusions
High levels of TNFRSF12A associated with MMP-9 overexpression may be important to explain the progression of breast cancer, and survival could be improved using therapy targeting TNFRSF12A.

Introduction
Breast cancer is one of the most common types of cancer diagnosed and main cause of death from cancer among women (23% of all cancer cases, 14% of deaths from cancer) [1]. Invasive ductal carcinoma (IDC) represents 70 to 80 percent of all breast cancer cases, followed by lobular carcinoma, tubular/cribriform carcinoma, mucinous carcinoma, medullary carcinoma, and papillary carcinoma [2]. Several findings, such as cancer development, cancer stage, oestrogen receptor (ER) and progesterone receptor (PR) expression, human epithelial growth factor receptor 2 (HER2) gene amplification, p53 overexpression, and BRCA1 and BRCA2 mutations, are taken into account in the development, treatment, and management of breast cancer [3]. Recently, in molecular subtyping using the above markers, breast cancer was classified into five major subtypes: luminal A, luminal B HER2-negative, luminal B HER2-positive, basal-like, and HER2-positive [4].

Matrix metalloproteinases (MMPs) is critical in decomposition of the extracellular membrane (ECM), including the basement membrane, a specialized matrix comprising type IV collagen, laminin, entactin, proteoglycans, and glycosaminoglycans [5]. Twenty-five MMPs have been identified to play critical roles in cancer progression via many carcinogenic processes, including remodelling of the microenvironment. Recently, studies on treatments targeting MMP-2 have also been reported [6,7].

MMP-9, a member of the MMPs, has long been postulated to play an important role in poor prognosis in tumour cells [8]. MMP-9 causes the release of the biologically active form of vascular endothelial growth factor, which is critical in angiogenesis [9,10]. The process is completed by the direct proteolytic degradation of vascular basement membrane proteins, meaning that MMP-9 can be essential in the creation of new blood vessels [10]. MMP-9 degrades the collagen-rich ECM, which has been related to cancer invasiveness, metastasis, and recurrence [11,12]. Therefore, MMP-9 has been considered an important prognostic predictor in various tumours.

In previous studies, MMP-9 was reported to be overexpressed in aggressive tumours [13–15]. Additionally, MMP-9 is required for endothelial cell migration and tube formation, and is likely to be important in cerebral angiogenesis [16]. Thus, an increase in MMP-9 levels associated with vascular angiogenesis could promote tumour invasion and metastasis. MMP-9 is involved in a variety of developmental processes because of its important role in angiogenesis. MMP-9 deficiency leads to bone development disorders, particularly bone formation delay caused by insufficient angiogenesis in the growth plate [17]. Recent studies demonstrated that MMP-9 could be involved in natural killer (NK) cell dysfunction by promoting tumour immune avoidance. Hence, the effect of NK cell-based immunotherapy could be enhanced by controlling MMP-9 activity [18].
The present study aimed to assess whether MMP-9 levels could predict patient survival and to analyse its prognostic value in comparison with the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) database. We further aimed to investigate the prognostic utility of MMP-9 in two cohorts (our data and METABRIC data), and to identify genes associated with MMP-9 overexpression using gene set enrichment analysis (GSEA). In addition, survival analysis was performed to confirm the clinical significance of the newly identified genes (Fig 1).

Materials and methods

Patient selection

This study included patients who were treated with breast cancer at Kangbuk Samsung Medical Center in Korea between 2000 and 2005. In randomly selected patients, immunohistochemical staining was performed on 173 specimens obtained from surgical treatment. The Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria were followed throughout this study. The inclusion criteria were: 1) patients with histopathological evidence of primary invasive ductal carcinoma confirmed by pathologists, and known clinical outcome; and 2) patients without preoperative concurrent chemoradiotherapy.

The mean age of the patients was 47 (range: 25–79) years. Treatments comprised 163 (94.2%) modified radical mastectomies with axillary lymph node dissection and 10 (5.8%) breast-conserving surgeries. At the time of surgery, the T and N stages, according to the 8th American Joint Committee on Cancer (AJCC), were distributed as follows: T1, 61 (35.3%); T2, 100 (57.8%); T3, 11 (6.4%); T4, 1 (0.6%); N0, 68 (39.3%); N1, 62 (35.8%); N2, 23 (13.3%); and N3, 20 (11.6%). The distribution of AJCC stages was as follows: stage I, 31 (17.9%); stage II, 97 (56.1%); and stage III, 45 (26%).

Haematoxylin and eosin (H&E)-stained slides were reviewed by at least two pathologists for each case (Min and Kim). The tumours were characterized according to their clinicopathological features, such as size, margins, number of tumours (single or multiple), histological grade, necrosis, and tumour fibrosis. Cases with unavailable paraffin blocks and inadequate clinical history were excluded. The histological grade was determined according to the modified Bloom–Richardson–Elston grading system [19].

This study (involving human participants) was approved by the Ethics Committee of the Kangbuk Samsung Hospital, Seoul, Republic of Korea (KBSMC 2018-02-026) and performed according to the ethical standards of the Declaration of Helsinki, as revised in 2008. The review conducted by our institutional review board confirmed that informed consent was not necessary for this study.

Tissue microarray construction and immunohistochemistry

The most morphologically representative and non-necrotic areas were carefully selected and marked on the H&E-stained slides. The tissue microarray (TMA) specimens were assembled using a tissue-array instrument (AccuMax Array; ISU ABXIS Co. Ltd, Seoul, Korea) consisting of thin-walled stainless steel punches and stylets for emptying and transferring the needle’s content. The assembly was held in an X-Y position guide equipped with semiautomatic micrometres, with a 1-mm increment between the individual samples and a 4-mm punch depth stop device. The instrument was briefly used to create holes in a recipient block with defined array cores. The micro-needle was used to transfer the tissue cores into the recipient block. Taking into account the limitations of the representative areas of the tumour, we used duplicate 3-mm-diameter tissue cores from each donor block. The percentage of tumour in the tissue cores was greater than 70%.
Protein expression using immunohistochemistry

MMP-9 staining in breast cancer tissue

Validation of Clinical significance of MMP-9

Survival analyses
- Our cohort
- METABRIC cohort

Identification of TNFRSF12A associated with MMP-9

Using gene set enrichment analysis in METABRIC cohort

Evaluation of Clinical significance of TNFRSF12A

Survival analyses
- METABIC cohort
- GSE1456 cohort
- GSE20685 cohort
Serial sections, 4-μm each, were cut from the array blocks and deparaffinised using routine techniques. Immunostaining for PR, ER, HER2, and marker of proliferation Ki-67 (Ki-67) was performed using the Dako Autostainer Universal Staining System (DakoCytomation, Carpinteria, CA, USA), and the ChemMate DAKO EnVision Detection Kit. The following primary antibodies were used: anti-ER (1:200; Lab Vision Corporation, Fremont, CA, USA), anti-PR (1:200; Dako, Glostrup, Denmark), anti-HER2 (1:200; Dako), and anti-Ki-67 (1:200; Dako). Immunostaining was used to classify the five molecular subtypes of breast cancer: luminal A (ER-positive and/or PR-positive; HER2-negative; Ki-67 low), luminal B HER2-negative (ER-positive and/or PR-positive; HER2-negative; Ki-67 high), luminal B HER2-positive (ER-positive and/or PR-positive; HER2-positive), HER2-positive (ER-negative and PR-negative; HER2-positive), and triple-negative (ER-negative and PR-negative; HER2-negative) [4].

For MMP-9, affinity-purified rabbit anti-human MMP-9 polyclonal antibodies (1:100; NeoMarkers Corporation, Fremont, CA, USA) were used, and detection was performed using the Ultra Tech HRP Streptavidin-Biotin Detection System (Beckman Coulter, Marseille, France) with an automatic staining machine (Bond Intense Detection Kit; Leica Biosystems, Newcastle, UK). Positive MMP-9 immunostaining was defined as exclusively cytoplasmic staining without nuclear staining, and was graded according to the intensity of staining of tumour cells. Staining intensity was scored on a scale of 0 to 3 (0 = negative; 1 = weak; 2 = moderate; 3 = strong) (Fig 2). MMP-9 expression was determined as either negative (intensity ≤ 1) or positive (intensity > 1) using the receiver operating characteristic (ROC) curve.

Data extraction and GSEA from the METABRIC database
Gene data (cDNA microarray profiling, Illumina HT-12 v3 platform) from METABRIC were downloaded from the domain training data (www.cbioportal.org/) [20]. MMP9 expression and clinicopathological data (AJCC, tumour size, histological grade, ER, PR, HER2, and overall survival) were extracted using the R package (http://www.r-project.org/). Normal samples as well as tumour samples with missing data were excluded from analysis.

GSEA is a method of analysing and interpreting microarray and other such data based on biological information. These biological sets can be published information about a biochemical

![Fig 1. A schematic diagram depicting the analysis pipeline in our study.](https://doi.org/10.1371/journal.pone.0202113.g001)

![Fig 2. Representative photomicrographs showing MMP-9 expression of weak (A), moderate (B), and strong (C) intensity by immunohistochemical staining in invasive ductal carcinoma (original magnification, ×400).](https://doi.org/10.1371/journal.pone.0202113.g002)
pathway or coexpression obtained in a previous experiment. GSEA was performed using GSEA version 3.0 from the Broad Institute at MIT (software.broadinstitute.org/gsea/index.jsp/).

The parameters used for the analysis were as follows. The data set had 17,786 features in the Molecular Signatures Database (MSigDB). To identify the oncogenic signatures that are significantly enriched in genes associated with high MMP9 expression, a gene set database (c6.all.v6.1symbols.gmt including 189 gene sets) was used for GSEA, with 1000 permutations used to calculate the p values, and permutation type was set to phenotype. We defined a meaningful gene set as one with a false discovery rate (FDR) of < 0.2, a family wise-error rate (FWER) of < 0.4, and p < 0.05.

Gene expression profiling data sets such as GSE1456 and GSE20685 were downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). In three independent cohorts, including METABRIC, GSE1456, and GSE20685, survival analyses were performed to verify the clinical significance of meaningful genes identified using GSEA.

**Statistical analysis**

Correlations between clinicopathological parameters and MMP-9 expression were analysed using the χ² test and a linear-by-linear association test. Disease-free survival (DFS) was defined as survival from the date of diagnosis to recurrence/new distant metastasis, and overall survival (OS) was defined as survival from the date of diagnosis to cancer-related death. Survival curves were generated using the Kaplan–Meier method, applying the cut-off values calculated from the ROC, and then compared using the log rank test. Multivariate analysis was performed to identify independent prognostic markers for DFS and OS using a Cox multistep regression model. A two-tailed p value of < 0.05 was considered statistically significant. All data were analysed using SPSS statistics for Windows version 24.0 (SPSS Inc., Chicago, IL, USA) or the R package (http://www.r-project.org/).

**Results**

**Clinicopathological parameters according to MMP-9 expression**

In the breast cancer cases from our hospital, high MMP-9 expression was significantly associated with high histological grade. In the METABRIC cases, high MMP-9 expression was significantly associated with high histological grade, ER/PR negativity, and HER2 positivity (all p < 0.001) (Table 1). In both datasets, high MMP-9 expression was more frequently observed in patients with advanced AJCC stage. However, the relationships were not statistically significant (p = 0.080 and 0.473 for our data and the METABRIC data, respectively). High MMP-9 expression was more frequently observed in the HER2 subtype than in the luminal A/B or triple-negative subtype (p = 0.024) (Table 2, Fig 3).

**Survival analyses according to MMP-9 expression**

In our data, high MMP-9 expression was significantly correlated with poor DFS and OS (p = 0.001 and 0.001, respectively). After adjusting for confounders, including T and N stage, histological grade, lymphatic/perineural invasion, ER/PR and HER2, there was still a significant relationship between MMP-9 and DFS and OS (p = 0.004 and 0.011, respectively). In the METABRIC data, high MMP-9 expression was significantly associated with worse OS in univariate analysis (p = 0.004). After adjusting for confounders, including AJCC stage, histological grade, ER/PR and HER2, the association between high MMP-9 expression and worse OS remained significant (p = 0.027) (Table 3, Fig 4).
## Table 1. Clinicopathological parameters of MMP-9 from our data and METABRIC data.

| Parameter               | MMP-9 (our case) | p value | MMP-9 (METABRIC) | p value |
|-------------------------|------------------|---------|-----------------|---------|
|                        | Low (n = 92), n (%) | High (n = 81), n (%) | Low (n = 235), n (%) | High (n = 1118), n (%) |
| AJCC                    |                  |         |                 |         |
| I                       | 13 (14.1)        | 18 (22.2) | 0.080<sup>a</sup> | 85 (36.2) | 370 (33.1) | 0.473<sup>a</sup> |
| II                      | 55 (59.8)        | 42 (51.9) | 132 (56.2) | 646 (57.8) |
| III                     | 24 (26.1)        | 21 (25.9) | 16 (6.8)   | 95 (8.5)   |
| IV                      | -                | -        | 2 (0.9)    | 7 (0.6)    |
| Tumour size             |                  |         |             |         |
| ≤2 cm                   | 34 (37.0)        | 28 (34.6) | 0.744       | 109 (46.4) | 499 (44.6) | 0.624 |
| >2 cm                   | 58 (63.0)        | 53 (65.4) | 126 (53.6) | 619 (55.4) |
| Histological grade      |                  |         |             |         |
| 1                       | 14 (15.2)        | 12 (14.8) | <0.001<sup>b</sup> | 36 (15.3) | 78 (7)       | 0.001<sup>b</sup> |
| 2                       | 49 (53.3)        | 30 (37.0) | 132 (56.2) | 401 (35.9) |
| 3                       | 29 (31.5)        | 39 (48.1) | 67 (28.5)  | 639 (57.2) |
| Lymphatic invasion      |                  |         |             |         |
| Negative                | 46 (50.0)        | 32 (39.5) | 0.166       | -        | -           |
| Positive                | 46 (50.0)        | 49 (60.5) | -           | -        |
| Perineural invasion     |                  |         |             |         |
| Negative                | 79 (85.9)        | 70 (86.4) | 0.917       | -        | -           |
| Positive                | 13 (14.1)        | 11 (13.6) | -           | -        |
| Tumour necrosis         |                  |         |             |         |
| Absence                 | 49 (5.3)         | 45 (55.6) | 0.762       | -        | -           |
| Presence                | 43 (46.7)        | 36 (44.4) | -           | -        |
| ER                      |                  |         |             |         |
| Negative                | 27 (29.3)        | 33 (40.7) | 0.116       | 14 (6)   | 299 (26.7) | <0.001 |
| Positive                | 65 (70.7)        | 48 (59.3) | 221 (94)   | 819 (73.3) |
| PR                      |                  |         |             |         |
| Negative                | 41 (44.6)        | 43 (53.1) | 0.263       | 72 (30.6) | 577 (51.6) | <0.001 |
| Positive                | 51 (55.4)        | 38 (46.9) | 163 (69.4) | 541 (48.4) |
| HER2                    |                  |         |             |         |
| Negative                | 71 (77.2)        | 55 (67.9) | 0.171       | 221 (94) | 964 (86.2) | 0.001 |
| Positive                | 21 (22.8)        | 26 (32.1) | 14 (6.0)   | 154 (13.8) |

AJCC, American Joint Committee on Cancer, 8<sup>th</sup> edition; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; MMP, matrix metalloproteinase; PR, progesterone receptor.

<sup>a</sup>AJCC stage I/II versus III.

<sup>b</sup>Histological grade 1/2 versus 3.

Bold indicates p < 0.05.

https://doi.org/10.1371/journal.pone.0202113.t001

## Table 2. MMP-9 expression according to molecular subtype.

| MMP-9         | Luminal A | Luminal B HER2- | Luminal B HER2+ | HER2 | Triple-negative | p value |
|---------------|-----------|-----------------|-----------------|------|-----------------|---------|
| Low           | 53 (56.4) | 3 (75)          | 9 (60)          | 9 (33.3) | 18 (54.5) | 0.024<sup>a</sup> |
| High          | 41 (43.6) | 1 (25)          | 6 (40)          | 18 (66.7) | 15 (45.5) |         |

HER2, human epidermal growth factor receptor 2; MMP, matrix metalloproteinase.

<sup>a</sup>Comparison of MMP-9 expression between HER2 and other subtypes.

Bold indicates p < 0.05.

https://doi.org/10.1371/journal.pone.0202113.t002
Identification of the association of TNFRSF12A associated with high MMP-9 expression

We conducted GSEA to identify the genes associated with high MMP-9 expression and found eleven significantly enriched gene sets: RB_P107_DN.V1_UP, IL15_UP.V1_UP, ATF2_S_UP.V1_UP, ATF2_UP.V1_UP, CSR_LATE_UP.V1_UP, P53_DN.V2_UP, E2F1_UP.V1_UP, MTOR_UP.V1_UP, IL2_UP.V1_UP, PKCA_DN.V1_DN, and BMI1_DN_MEL18_DN. We found that TNFRSF12A (encoding tumour necrosis factor receptor superfamily member 12A) was common in four gene sets (IL15_UP.V1_UP, MTOR_UP.V1_UP, IL2_UP.V1_UP, PKCA_DN.V1_DN, and BMI1_DN_MEL18_DN. V1_UP (Table 4). We found that TNFRSF12A (encoding tumour necrosis factor receptor superfamily member 12A) was common in four gene sets (IL15_UP.V1_UP, MTOR_UP.V1_UP, IL2_UP.V1_UP, and PKCA_DN.V1_DN). Survival analyses were performed in three data sets (METABRIC, GSE1456, GSE20685) to determine the significance of TNFRSF12A expression levels. High TNFRSF12A expression was significantly associated with worse OS (all \( p < 0.05 \)) (Fig 5).

Discussion

In cancer processes, the activities of certain extracellular matrix degradative enzymes provide favourable conditions for tumour growth and subsequent dissemination and metastasis. This study evaluated MMP-9 expression in relation to various clinicopathological parameters in breast cancer. Interestingly, our results revealed that high MMP-9 expression is associated
with a high histological grade and HER2 subtype, as well as worse DFS and OS in patients with breast cancer. To further reinforce the implications of these findings, we reanalysed the association of MMP-9 with clinical data from METABRIC, a large-scale study, to improve the reproducibility of the findings. As in our study, high MMP-9 expression was associated with poor

| Survival                  | Univariate | Multivariate | HR     | 95% CI    |
|---------------------------|------------|--------------|--------|-----------|
| Disease-free survival     |            |              |        |           |
| MMP-9 (low vs. high)      | 0.001      | 0.004        | 10.901 | 2.124     | 55.950    |
| T stage (1 or 2 vs. 3)    | 0.649      | 0.201        | 4.477  | 0.449     | 44.641    |
| N stage (0, 1 or 2 vs. 3) | 0.147      | 0.122        | 3.393  | 0.720     | 15.989    |
| Histologic grade (1 or 2 vs. 3) | 0.039 | 0.615        | 1.429  | 0.356     | 5.740     |
| Lymphatic invasion (negative vs. positive) | 0.515 | 0.198        | 0.407  | 0.104     | 1.598     |
| Perineural invasion (negative vs. positive) | 0.013 | 0.023        | 4.181  | 1.215     | 14.386    |
| ER/PR (positive vs. negative) | 0.05    | 0.342        | 1.985  | 0.483     | 8.164     |
| HER2 (negative vs. positive) | 0.105     | 0.354        | 1.803  | 0.519     | 6.263     |
| Overall survival          |            |              |        |           |
| MMP-9 (low vs. high)      | 0.011      | 0.011        | 3.720  | 1.354     | 10.221    |
| T stage (1 or 2 vs. 3)    | <0.001     | 0.002        | 5.894  | 1.926     | 18.034    |
| N stage (0, 1 or 2 vs. 3) | <0.001     | 0.002        | 6.047  | 1.975     | 18.516    |
| Histologic grade (1 or 2 vs. 3) | <0.001 | 0.690        | 1.273  | 0.389     | 4.170     |
| Lymphatic invasion (negative vs. positive) | <0.001 | 0.276        | 2.125  | 0.547     | 8.254     |
| Perineural invasion (negative vs. positive) | 0.015 | 0.106        | 2.203  | 0.845     | 5.744     |
| ER/PR (positive vs. negative) | 0.003      | 0.364        | 1.693  | 0.543     | 5.277     |
| HER2 (negative vs. positive) | <0.001     | 0.016        | 3.487  | 1.264     | 9.617     |

T and N stages are according to the American Joint Committee on Cancer, 8th edition.
ER/PR, estrogen receptor/progesterone receptor; HER2, human epithelial growth factor receptor 2

*Log rank test.
*Cox proportional hazards model.
Bold indicates p < 0.05.

https://doi.org/10.1371/journal.pone.0202113.t003

Fig 4. Survival curves derived using the Kaplan–Meier method showing correlation with MMP-9. (A) Disease-free survival, (B) overall survival, (C) overall survival in the METABRIC data (p = 0.001, 0.011, and 0.002, respectively).

https://doi.org/10.1371/journal.pone.0202113.g004
clinical outcomes, such as high histological grade and lower OS. In addition, MMP-9 was significantly associated with the levels of ER, PR, and HER2, which are important factors in breast cancer treatment. Thus, MMP-9 might play a major role in promoting cancer progression, and the evaluation of enzymes like MMP-9 may be helpful in predicting clinical outcomes.

Many published studies have demonstrated that MMP-9 overexpression is associated with various malignant tumours [12,21,22]; however, its significance was not clear in other reports [23,24]. A study by Scorilas et al. demonstrated that MMP-9 overexpression is associated with a favourable prognosis in patients with breast cancer without lymph node metastasis [25]. A study by Pellikainen et al. suggested that MMP-9 expression in stromal cells is associated with poor prognosis in patients receiving hormonal therapy; however, MMP-9 expression in tumour cells indicated good prognosis [24]. Therefore, the patient’s prognosis differs depending on the specific state of the individual patient and whether MMP-9 expression occurs in the tumour or stromal cells. Therefore, controversy still exists regarding the relationship between MMP-9 expression and clinical outcomes in various malignant tumours. The difference in the prognostic value of MMP-9 expression in stromal and tumour cells might reflect the presence of active and inactive forms of MMP-9. Generally, MMPs are secreted as inactive proenzymes that are activated by many mechanisms, including organomercurials, chaotropic agents, and other proteases. The amount of the active form of MMP-9 in stromal cells and tumour cells may differ, which could account for the differences in clinical outcomes.

In our data, the expression of MMP-9 was significantly correlated with unfavourable clinicopathological parameters, even though its expression was unrelated to other parameters. Therefore, MMP-9 expression in tumour cells might play an important role in several prognostic processes. High MMP-9 expression was associated with poor OS, and the association remained significant after adjustment for potential confounders. Notably, our results, showing that high MMP-9 expression was associated with poor prognosis, were validated in a large-scale breast cancer cohort, METABRIC. Nevertheless, a dissociation between survival and MMP-9 is thought to derive from incredible advance of technical surgical procedures, chemotherapy, hormonal therapy, and the number of immune cells raised against tumour cells. In addition, the following factors can be considered as variables that can lead to conflicting results: study design, cancer type, ethnic factors, and the number of patients.

Table 4. Gene sets related to high MMP-9 expression.

| NAME                     | SIZE | ES     | NES   | NOM (p-value) | FDR (q-value) | FWER (p-value) |
|--------------------------|------|--------|-------|---------------|---------------|---------------|
| RB_P107_DN.V1_UP         | 134  | 0.667521| 1.7672| <0.000001     | 0.012797      | 0.006         |
| IL15_UP.V1_UP            | 166  | 0.632059| 1.738388| <0.000001     | 0.007296      | 0.007         |
| ATF2_S_UP.V1_UP          | 175  | 0.571068| 1.715975| <0.000001     | 0.00982       | 0.014         |
| ATF2_UP.V1_UP            | 170  | 0.566506| 1.700243| <0.000001     | 0.008313      | 0.017         |
| CSR_LATE_UP.V1_UP        | 162  | 0.645542| 1.675572| 0.003984      | 0.009363      | 0.026         |
| P53_DN.V2_UP             | 137  | 0.572954| 1.674256| <0.000001     | 0.008579      | 0.028         |
| E2F1_UP.V1_UP            | 179  | 0.559687| 1.645125| 0.001949      | 0.01346       | 0.053         |
| MTO1_UP.V1_UP            | 150  | 0.59151 | 1.632898| <0.000001     | 0.016188      | 0.068         |
| IL2_UP.V1_UP             | 168  | 0.570225| 1.604615| <0.000001     | 0.018264      | 0.096         |
| PKCA_DN.V1_DN            | 151  | 0.547922| 1.59843| <0.000001     | 0.018233      | 0.105         |
| BMI1_DN_MEL18_DN.V1_UP   | 133  | 0.627022| 1.551241| 0.002004      | 0.019859      | 0.185         |

ES, Enrichment Score; NES, Normalized Enrichment Score; FDR, False Discovery Rate; NOM, Nominal p Value; FDR, False discovery rate; FWER, Family wise-error rate

https://doi.org/10.1371/journal.pone.0202113.t004
We identified TNFRSF12A as an MMP-9-related gene in patients with breast cancer. TNFRSF12A is one of the TNF ligand family that induces angiogenesis in vivo [26]. In the GEO database, the survival rate of patients in association with TNFRSF12A levels was analysed to determine its clinical significance. High levels of TNFRSF12A accompanied by MMP-9 overexpression were associated with poor OS. This result suggested that high TNFRSF12A expression is related to aggressive behaviour of breast cancer. The relationship between the MMP-9 and TNFRSF12A needs to be proven through experimentation; however, we speculated that TNFRSF12A associated with high MMP-9 expression might accelerate cancer progression through increased angiogenesis.

This study had some limitations that should be acknowledged. First, because our cross-sectional study did not show sustained relationships over time, as prospective studies do, it is difficult to come to a definitive conclusion. Further in vitro and/or in vivo studies of MMP-9

Fig 5. Gene expression data analysed using gene set enrichment analysis (GSEA) to extract biological information. In each thumbnail, the green curve represents the evolution of the density of the genes identified in the RNA-seq. GSEA calculates these by walking down the ranked-ordered list of genes, increasing a running-sum statistic when a gene is in the gene set and decreasing it when it is not. (A) TNFRSF12A was found commonly in four gene sets (IL15_UP.V1.UP, MTOUR_UP.V1.UP, IL2_UP.V1.UP and PKCA_DN.V1.DN) with high MMP-9 expression. (B) High TNFRSF12A was associated with poor prognosis in three independent cohorts (METABRIC, GSE1456, and GSE20685).

https://doi.org/10.1371/journal.pone.0202113.g005
expression may be necessary. Second, representative tissue may not have been assessed because it was included in only two sections of each tumour specimen. Third, alterations in MMP-9 expression could not be evaluated, because the level of MMP-9 expression before and after adjuvant therapy was not investigated.

Conclusions

In summary, the study showed that high MMP-9 expression is significantly associated with poor DFS and OS in two cohorts (our data and METABRIC data). High expression of TNFRSF12A, a gene associated with MMP-9 overexpression through GSEA, was shown to be associated with worse OS in three independent genetic data sets. The newly discovered gene, TNFRSF12A, could be an important factor in explaining breast cancer progression associated with MMP-9. In the future, targeted therapy for TNFRSF12A might improve the survival of patients with MMP-9 overexpression.

Author Contributions

Conceptualization: Kyung-Whan Min, Dong-Hoon Kim.
Data curation: Jungho Yang, Byoung Kwan Son, Kyoung Min Moon, Young Chan Wi, Seong Sik Bang, Young Ha Oh, Sung-Im Do, Seoung Wan Chae, Sukjoong Oh, Young Hwan Kim, Mi Jung Kwon.
Funding acquisition: Kyung-Whan Min.
Methodology: Kyung-Whan Min.
Supervision: Kyung-Whan Min.
Writing – original draft: Jungho Yang.
Writing – review & editing: Kyung-Whan Min.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61(2):69–90. https://doi.org/10.3322/caac.20107 PMID: 21296855
2. Page DL, Dixon JM, Anderson TJ, Lee D, Stewart HJ. Invasive cribriform carcinoma of the breast. Histo-pathology 1983; 7(4):525–36. PMID: 6884999
3. Parise CA, Bauer KR, Brown MM, Caggiano V. Breast cancer subtypes as defined by the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) among women with invasive breast cancer in California, 1999–2004. Breast J 2009; 15(6):593–602. https://doi.org/10.1111/j.1524-4741.2009.00822.x PMID: 19764994
4. Prat A, Carey LA, Adamo B, Vidal M, Tabernerio J, Cortés J, et al. Molecular features and survival outcomes of the intrinsic subtypes within HER2-positive breast cancer. J Natl Cancer Inst 2014; 106(8).
5. Jabłońska-Trypuć A, Matejczyk M, Rosochacki S. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. J Enzyme Inhib Med Chem 2016; 31(sup1):177–83. https://doi.org/10.3109/14756366.2016.1161620 PMID: 27028474
6. Giulelli C, Theocharis AD, Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FEBS J 2011; 278(1):16–27. https://doi.org/10.1111/j.1742-4658.2010.07919.x PMID: 21087457
7. Giussani M, Merlino G, Cappelletti V, Tagliabue E, Daidone MG. Tumor-extracellular matrix interactions: Identification of tools associated with breast cancer progression. Semin Cancer Biol 2015; 35:3–10. https://doi.org/10.1016/j.semcancer.2015.09.012 PMID: 26416466
8. Mehner C, Hockla A, Miller E, Ran S, Radisky DC, Radisky ES. Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. Oncotarget 2014; 5(9):2736–49. https://doi.org/10.18632/oncotarget.1932 PMID: 24811362
9. Zeng ZS, Cohen AM, Guillem JG. Loss of basement memhrane type IV collagen is associated with increased expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) during human colorectal tumorigenesis. *Carcinogenesis* 1999; 20(5):749–55. PMID: 10334190

10. Klein T, Bischoff R. Physiology and pathophysiology of matrix metalloproteinases. *Amino Acids* 2011; 41(2):271–90. https://doi.org/10.1007/s00726-010-0689-x PMID: 20640864

11. Shen Z, Zhu D, Liu J, Chen J, Liu Y, Hu C, et al. 27-Hydroxycholesterol induces invasion and migration of breast cancer cells by increasing MMP9 and generating EMT through activation of STAT-3. *Environ Toxicol Pharmacol* 2017; 51:1–8. https://doi.org/10.1016/j.etap.2017.02.001 PMID: 28257824

12. Tabouret E, Bertucci F, Pierga J-Y, Petit T, Levy C, Ferrero J-M, et al. MMP2 and MMP9 serum levels are associated with favorable outcome in patients with inflammatory breast cancer treated with bevacizumab-based neoadjuvant chemotherapy in the BEVERLY-2 study. *Oncotherapy* 2016; 7(14):18531–40. https://doi.org/10.18632/oncotarget.7612 PMID: 26921265

13. Puzovic V, Brcic I, Ranogajec I, Jakic-Razumovic J. Prognostic values of ETS-1, MMP-2 and MMP-9 expression and co-expression in breast cancer patients. *Neoplasma* 2014; 61(4):439–46. https://doi.org/10.4149/neo_2014_054 PMID: 24645837

14. Sillanpää S, Anttila M, Voutilainen K, Ropponen K, Turpeenniemi-Hujanen T, Puistola U, et al. Prognostic significance of matrix metalloproteinase-9 (MMP-9) in epithelial ovarian cancer. *Gynecol Oncol* 2007; 104(2):296–303. https://doi.org/10.1016/j.ygyno.2006.09.004 PMID: 17034838

15. Nart D, Yaman B, Yilmaz F, Zeytunlu M, Karasu Z, Kılıç M, et al. Expression of matrix metalloprotease-9 in predicting prognosis of hepatocellular carcinoma after liver transplantation. *Liver Transpl* 2010; 16(5):621–30. https://doi.org/10.1002/lt.22028 PMID: 20440771

16. Jadhav U, Chigurupati S, Laksha SS, Mohanam S. Expression of matrix metalloproteinase-9 in predicting prognosis of hepatocelellular carcinoma after liver transplantation. *Liver Transpl* 2010; 16(5):621–30. https://doi.org/10.1002/lt.22028 PMID: 20440771

17. Vu TH, Shipleym JM, Bergers G, Berger JE, Helms JA, Hanahan D, et al. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 1998; 93(3):411–22. PMID: 9990175

18. Peng Y-P, Zhang J-J, Liang W, Tu M, Lu Z-P, Wei J-S, et al. Elevation of MMP-9 and IDO induced by pancreatic cancer cells mediates natural killer cell dysfunction. *BMC Cancer* 2014; 14:738. https://doi.org/10.1186/1471-2407-14-738 PMID: 25274283

19. Robbins P, Pinder S, de Klerk N, Dawkins H, Harvey J, Sterrett G, et al. Histologic grading of breast carcinomas: a study of interobserver agreement. *Hum Pathol* 1995; 26(8):873–9. PMID: 7635449

20. Curtis C, Shah SP, Chin S-F, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012; 486(7403):346–52. https://doi.org/10.1038/nature10983 PMID: 22522925

21. Kodate M, Kasai T, Hashimoto H, Yasumoto K, Iwata Y, Manabe H. Expression of matrix metalloproteinase (gelatinase) in T1 adenocarcinoma of the lung. *Pathol Int* 1997; 47(7):461–9. PMID: 9234385

22. Papathomasis AS, Petraki C, Grigorakis A, Papakonstantinou H, Karavana V, Stefanakis S. Prognostic significance of matrix metalloproteinases 2 and 9 in bladder cancer. *Anticancer Res* 2000; 20(3B):2009–13. PMID: 10928143

23. Rahko E, Jukkola A, Melkko J, Paavo P, Bloigu R, Talvensaari-Mattila A, et al. Matrix metalloproteinase-9 (MMP-9) immunoreactive protein has modest prognostic value in locally advanced breast carcinoma patients treated with an adjuvant antiestrogen therapy. *Anticancer Res* 2004; 24(6):4247–53. PMID: 15736480

24. Pellikainen JM, Ronponen KM, Kataja KV, Kellokoski JK, Eskelinen MJ, Kosma V-M. Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in breast cancer with a special reference to activator protein-2, HER2, and prognosis. *Clin Cancer Res* 2004; 10(22):7621–8. https://doi.org/10.1158/1078-0432.CCR-04-1061 PMID: 15569994

25. Scorilas A, Karameris A, Arnogiannaki N, Ardavanis A, Bassilopoulos P, Trangas T, et al. Overexpression of matrix-metalloproteinase-9 in human breast cancer: a potential favourable indicator in node-negative patients. *Br J Cancer* 2001; 84(11):1488–96. https://doi.org/10.1054/bjc.2001.1810 PMID: 11384099

26. Wiley SR, Cassiano L, Lofton T, Davis-Smith T, Winkles JA, Lindner V, et al. A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. *Immunity* 2001; 15(5):837–46. PMID: 11728344