Research Paper:
The Gene Expression of *MMP-2* and *TIMP-1* in Non-pathological Paratumoral and Autopsy Lung Tissues

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ABSTRACT

Background: Finding a sample of healthy tissue is a critical challenge in research studies. Non-pathological Tissue adjacent to the tumor (NAT) specimens is usually used as the control in several studies. However, little is known about the similarity of NAT to healthy tissues. Here, we compared the expression of Matrix Metalloproteinase 2 (MMP-2) and its inhibitor, Tissue Inhibitors of MMP (TIMP)-1 as extracellular matrix remodeling factors in NAT and autopsy lung tissue.

Materials and Methods: RNA of 7 NAT and 6 Formalin-Fixed Paraffin-Embedded (FFPE) lung autopsies from healthy people as the control group was extracted, and cDNA was synthesized. The gene expression levels of *MMP-2* and *TIMP-1* were evaluated by real-time PCR.

Results: There were no significant differences in the expression of *MMP-2, TIMP-1*, or their ratio between the two groups.

Conclusion: The results showed that NAT could be used as healthy controls in lung tissue studies for *MMP-2* and *TIMP*-1.

1. Introduction

The use of human tissue samples plays a vital role in the development of physiologic and pathological research and current medical care [1]. Although blood, urine, or fecal samples provide us information on what happens in the body, none of these samples can be an accurate reflection of tissue events, and in some cases, their results differ from changes in local tissue. For example, Shabnam Fahim et al. showed decreased Angiotensin-Converting Enzyme (ACE) tissue level and increased ACE serum level in patients with alopecia areata [2]. In another study, Ginath et al. reported that the percentage of positive CA125 tissue staining is significantly higher than the increase in CA125 serum levels in endometrial cancer patients [3].

In research studies, a series of healthy control samples are always needed to compare with pathological speci-

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mens to examine the changes caused by pathological conditions properly. However, it is immoral to take normal tissue from healthy people, and in these cases, samples such as autopsy specimens, a part of transplanted organ tissue, or archival clinical specimens are employed. The Non-pathological Tissue Adjacent to the Tumor (NAT) is the available samples used as a control in research studies, especially in oncology [1]. However, the number of studies that compared the NAT with healthy tissues is limited. Notably, our knowledge comes from breast tissue, where healthy marginal tissue samples can be easily achieved from reduction mammoplasty and prophylactic mastectomy.

In 2017, Dvir Aran et al. used RNA-seq collection of the Genotype-Tissue Expression (GTEx) program and the Cancer Genome Atlas (TCGA) to conduct a comprehensive study on the expression of a large number of genes in the healthy autopsy, tumor, and NAT samples in eight tissues (uterus, bladder, breast, prostate, thyroid, lung, colon, and liver). This study explored that tumor-secreted factors affect the adjacent microenvironment to boost tumor metastasis. They suggested a pan-cancer mechanism of proinflammatory signals but not a repair process in NAT [4].

Several studies have focused on the tissue repair process and Extracellular Matrix (ECM) remodeling underlying the pathogenesis of chronic diseases. Matrix Metalloproteinases (MMP) are endopeptidases that need zinc or calcium atoms to function properly. These enzymes play diverse functions in the human body before and after birth in normal and pathological conditions [5]. Degradation of the lung ECM is necessary for development, wound healing, and lung repair after lung injury, which is performed by the MMPs [6]. MMPs are involved in the pathogenesis of emphysema, chronic obstructive pulmonary disease, and asthma [7]. Tissue Inhibitors of MMPs (TIMPs) are endogenous and natural inhibitors of MMPs [8]. A change in TIMP or MMP level can disrupt the MMP/TIMP ratio and cause pulmonary ECM remodeling in the lung [9].

The present study aimed to examine this hypothesis whether gene expression of *MMP-2* and *TIMP-1* as ECM remodeling factors are different between non-pathological paratumoral and autopsy lung tissue. For this reason, RNA isolated from both sample types was surveyed using real-time PCR as a standard gold technique.

2. Materials and Methods

Study design and materials

Out study samples included 30 Formalin-Fixed Paraffin-Embedded (FFPE) NAT tissue blocks from patients with lung cancer receiving surgery for diagnosis and 30 FFPE autopsy lung tissue blocks from healthy people who died because of accident or heart attack as the control group. The participants were 30 to 60 years old. They were no current smoker or addicted to drugs. Twenty non-pathological parts of NAT (with no inflammation and neoplasia) and 20 autopsy samples (with no signs of edema, hemorrhage, and inflammation) were selected by three tainted pathologists. However, because of the low quality of some RNA isolates, 7 samples in the autopsy group and 6 samples in the NAT group remained.

RNA extraction

Four 10-µm sections from each FFPE specimen were deparaffinized twice using xylene at 56°C for 30 min with 400 rpm agitation. The remaining xylene was washed twice by 96% ethanol. Total RNA was extracted according to the kit protocol (RNeasy FFPE Kit, QIAGEN). Unlike kit protocol, samples were incubated with proteinase K for 3 h. Immediately after extraction, the purity and concentration of RNA were evaluated by the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The samples with an A260/A280 ratio of less than 1.8 were excluded from the study. The integrity of RNA samples was appraised by Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany). The samples with DV200 (percentage of RNA fragments that are more than 200 nucleotides in size) of more than 30% were accepted. RNA (1000 ng) samples were transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA).

Quantitative Reverse Transcription-Polymerase chain Reaction (QRT-PCR)

The characteristics and specificity of designed primers (Table 1) were checked by online OligoAnalyzer™ Tool software and the Basic Local Alignment Search Tool, respectively. Real-time PCR was performed in triplicate using RealQ Plus 2x Master Mix Green (Amplicon, Denmark) on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). Each reaction contained 10 µL of 2X master mix, 6 µL nuclease-free water, 10 pmol from each primer (~ 1 µL), and 2 µL from 1: 2 diluted cDNA. The amplification program was as follows: initial denaturation at 95°C for 15 min, 50 cycles of two-step

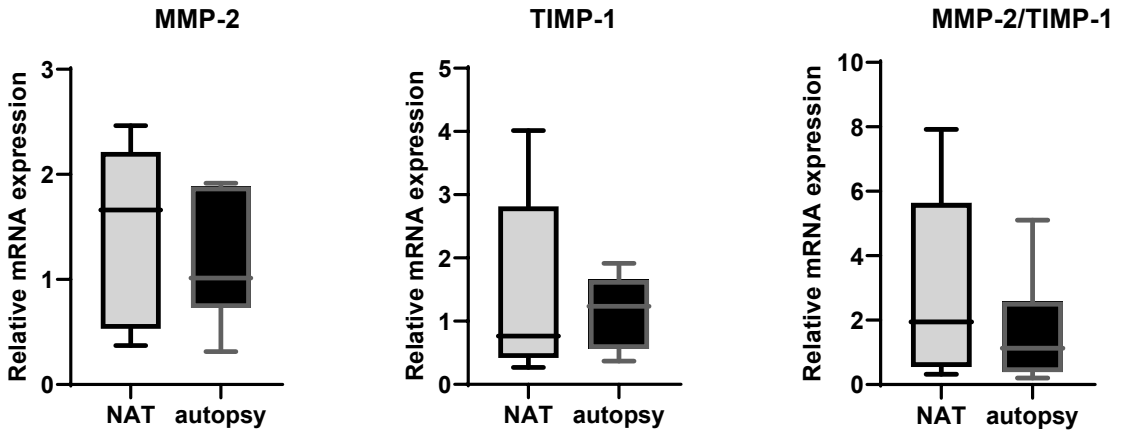

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Figure 1. The gene expression of *MMP-2, TIMP-1*, and the ratio of *MMP-2* to *TIMP-1* in the Non-pathological Tissue Adjacent to the Tumor (NAT) and the autopsy lung tissues

MMP-2 and *TIMP-1* were quantified in NAT (n=6) and health lung autopsy (n=7). The gene expression data were normalized using PGK1 as the reference gene. The obtained data were compared by the Mann-Whitney U test. The center of boxplots represents the median of the measurement, and the first and third percentile are shown by the lower and upper bounds of the box. Whiskers beyond these points represent 1.5 × the interquartile range. P value <0.05 was considered statistically significant.

amplification, including 30 s denaturation at 95°C, and annealing at 60°C for 1 min. This procedure followed a melt curve resolution ramping 60°C to 95°C with a rate of 0.3°C per second. The data were normalized to the Phosphoglycerate Kinase1 (PGK1) level. Primers efficiency was computed using LinRegPCR software, version 2016.1.

Statistical analysis

The threshold cycle (CT) value of each sample was defined by StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). Because of high and uniform efficiency, we used the $2^{-\Delta\Delta Cq}$ (Livak) method for statistical analysis. The Mann-Whitney U test was used for mean comparison between groups. P value less than 0.05 was considered statistically significant. All statistical analyses were carried out using SPSS version 16.

3. Results

In this study, 7 FFPE NAT and 6 FFPE autopsy lung tissues were evaluated. There was no significant difference between the average age of NAT (36.86±10.19) and autopsy 45.83±6.88 groups (P=0.101). There were three males and four females in the NAT group, while all participants in the autopsy group were male.

The result demonstrated no significant differences in the expression of *MMP-2* (median: 1.661, Q1: 0.5322, Q3:2.214 vs median: 1.015 Q1: 0.7285, Q3: 1.883, P=0.490), *TIMP1* (median: 0.763, Q1: 0.4201, Q3: 2.815 vs median: 1.235, Q1: 0.5625, Q3: 1.654, P=0.730) and the ratio of *MMP-2/TIMP1* (median: 1.945, Q1: 0.5433, Q3: 5.636 vs median: 1.129, Q1: 0.3894, Q3: 2.567, P=0.699) between NAT and autopsy samples (Figure 1).

Table 1. The characteristics of specific primer sequences for Phosphoglycerate Kinase 1 (*PGK1*), Matrix Metalloproteinase 2 (*MMP-2*), and Tissue Inhibitors of MMP 1 (*TIMP1*)

Gene	Forward (5'->3')	Temperature (°C)	Product Length (Base Pair)
MMP-2	Forward: GATCTTGACCAGAATACCATC Reverse: GCCAATGATCCTGTATGTG	53.5 53.51	129
TIMP1	Forward: AAGATGACCAAGATGTATAAAGG Reverse: GGGACCTGTGGAAGTATC	53.89 53.58	109
PGK1	Forward: GGAAGAAGGGAAGGGAAAAGATG Reverse: AAGCATCATTGACATAGACATC	58.98 54.34	116

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4. Discussion

The utilization of human tissue specimens has contributed to remarkable scientific and medical development. A healthy tissue as a control group is always needed to examine pathological tissues. The non-pathological residue part of archival specimens is the available and valuable samples as a control group, especially in cancer research [1]. A comprehensive study demonstrated that NAT has an intermediate characteristic between tumor and healthy tissue [4]. They examined the alteration of gene expression using RNA-seq, and it remains to be validated by real-time PCR. This study aimed to check whether the expression of ECM remodeling agents such as *MMP-2* and *TIMP-1* are different between NAT and healthy autopsy specimens. We found no changes in the gene expression pattern of *MMP-2, TIMP*-1, and their ratio between the two groups.

Aran et al. using RNA seq. data collection reported that the expression of *MMP-2* (0.8 fold change) and *TIMP-1* (2.5 fold change) significantly downregulated in NAT compared with healthy control lung tissue [4]. In lung tissue studies, factors such as smoking or drug addiction are in the exclusion criteria [10], but Dvir Aran et al. had not mentioned any inclusion or exclusion criteria in their report [4]. In this study, we considered almost all factors that can affect lung tissue and cause incorrect conclusions, such as lung disease, smoking, or drug addiction. In addition, Trujillo et al. using microarray and real-time PCR, demonstrated that mean transcript level for MMP-2 differed between histologically normal breast tissues located 1 cm from the tumor margin (TAHN-1) compared with TAHN-5 tissues and reduction mammoplasty samples, but was not statistically significant [11]. Therefore, the tissues located more than 5 cm from the tumor margin are more similar to the healthy tissues.

A major limitation to investigate the gene expression was the degradation of RNA isolated from FFPE samples. Although the archival specimens are available in large numbers, the integrity of extracted RNA is extremely poor. Therefore, to perform the test on an acceptable number of samples, multi-center work must be done.

5. Conclusion

In conclusion, we could suggest that non-pathological tissue adjacent to the tumor can be used as the healthy control in lung tissue studies for MMP-2 and TIMP-1. However, evaluating several ECM remodeling factors in a larger sample size regarding the distance of tissue adjacent from the tumor margin could provide a better understanding of the difference between NAT and healthy lung tissue.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Immunoregulation Research Center and Research Ethics Committees of Shahed University (Code: IR.SHAHED.REC.1398.102).

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Authors' contributions

Conceptualization: Tooba Ghazanfari; Methodology: Sara Ghaffarpour, Elaheh Esmaeili, Alireza Sadeghipour; Investigation: All author; Writing original draft: Sara Ghaffarpour, Elaheh Esmaeili; Writing review & editing: Tooba Ghazanfari; Funding acquisition: Tooba Ghazanfari; Resources: Tooba Ghazanfari; Supervision: Tooba Ghazanfari.

Conflicts of interest

The authors declared no conflict of interest.

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