RESEARCH ARTICLE

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Long-Term Effect of *TIMP3* Gene Expression on Thyroid Cancer: A Cure Model Analysis

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Abstract

Background: Thyroid cancer is the most common endocrine malignancy. TIMP3, a metalloproteinase inhibitor, can inhibit angiogenesis, invasion, and metastasis in thyroid cancer. In this study, we investigated the long-term effect of *TIMP3* gene expression and other associated factors on the survival rate and cure probability of thyroid cancer patients. **Methods:** In this historical cohort study, clinical information was collected from 507 thyroid cancer patients and 59 control samples based on the TCGA database. The Kaplan-Meier curve and log-rank test were employed for group comparisons. Weibull mixture and non-mixture cure models were utilized to explore the association between *TIMP3* gene expression and survival time, as well as cure status. All statistical analyses were conducted using the R language. **Results:** There were 507 thyroid cancer patients and 59 normal tissue participants in the study with an average age of 47.93±15.96 and 47.06 ± 17.74 years respectively. A total of 26.8 percent of patients were male, 69.6 percent had high expression, and 3.16 percent died during the study. Compared with normal tissue participants, tumor-positive patients had significantly lower *TIMP3* expression ($p<0.001$). After 2,000 days of follow-up, 78 percent of patients were cured based on Kaplan-Meier curves. The results show that the Weibull mixture cure model is superior to the non-mixture cure model. Moreover, after controlling for other factors, higher *TIMP3* expression was associated with an increased chance of long-term recovery in patients. Specifically, the odds of cure in patients with higher *TIMP3* expression were approximately 2.3 times greater than others. **Conclusions:** *TIMP3* expression has a protective effect on cure probability in thyroid cancer patients, even though it does not appear to affect short-term survival. This study suggests that targeting *TIMP3* may offer promise for thyroid cancer and may be a potential biomarker for thyroid cancer prognosis.

Keywords: Thyroid cancer- *TIMP3* gene expression- Weibull mixture cure model- Weibull non-mixture cure model

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Introduction

Papillary thyroid cancer is the most common malignancy among thyroid diseases, comprising approximately 80% of thyroid cancers[1]. Thyroid cancer can be classified into well-differentiated thyroid cancer (WDTC), including papillary (PTC) at 80%, follicular (FTC) at 10 to 15%, and poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC), with a small percentage belonging to the latter two categories[2-4]. The annual mortality rate of thyroid cancers ranges from 0.2 to 1.2 per 100,000 in men and 0.4 to 2.8 per 100,000 in women[5]. In 2014, a total of 1,665,540 new cancer cases and 585,720 cancer deaths were reported in the United States, with 62,980 cases of thyroid cancer and 1,890 deaths[6, 7].

The general role of proteases, especially Matrix Metalloproteinases (MMPs), in tumor cell metastasis and invasion has been well-established. A crucial balance between MMPs and TIMPs (Tissue Inhibitors of Metalloproteinases) is essential, and an imbalance between the two is observed in the invasion and metastases of malignant tumors[8, 9]. It's important to note that the association between MMPs and TIMPs doesn't consistently correlate with increased tumorigenicity and invasiveness; high TIMP levels can also inhibit tumor growth[10].

Among the four known tissue metalloproteinase inhibitors, *TIMP3* plays a significant role in curtailing angiogenesis, invasion, and metastasis across various human cancers[11, 12]. Notably, studies involving tumor cell transplantation have shown that *TIMP3* deficiency in the host is sufficient to delay the growth of primary tumor cells, highlighting the importance of the *TIMP3* axis in inhibiting tumorigenesis [13, 14]. Proposed as a tumor suppressor in multiple human cancer types, the silencing of *TIMP3* through hypermethylation of its promoter is linked to a poor prognosis in cancers such as brain, colorectal

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and non-small cell lung [15-17]. For example, previous studies have shown that low expression of TIMP-3 is often associated with Increased tumor aggressiveness, Higher rates of metastasis and Poorer overall survival rates in breast cancer patients[18]. In other studies, *TIMP3* shows promise as a prognostic biomarker for gastric cancer and was associated with favorable overall survival [19].

In thyroid cancer, particularly in papillary thyroid carcinoma (PTC)m the *TIMP3* gene plays a vital role as an oncosuppressor. Its expression is frequently downregulated through promoter hypermethylation, contributing to aggressive tumor characteristics such as extrathyroidal invasion and lymph node metastasis, notably in BRAFV600E mutation cases[20, 12]. *TIMP3* inhibits critical processes like migration and angiogenesis in cancer cells, and restoring its expression has been linked to reduced tumor growth and macrophage infiltration. High *TIMP3* levels are associated with diminished inflammation and less aggressive tumor behavior, while low levels correlate with increased inflammation. Consequently, *TIMP3* is emerging as a promising therapeutic target for developing innovative treatments for aggressive thyroid cancer. Furthermore, both animal and human studies have demonstrated that *TIMP3* expression has a protective effect on the survival time of patients [21, 22]. However, studies on the effect of this gene on survival of patients with thyroid cancer are limited.

Consequently, this study was conducted to evaluate the impact of *TIMP3* expression on the survival time of individuals with thyroid cancer. Survival analysis models are methods that deal with modeling the time to an event in the presence of incomplete data (censored observations). Different from common statistical models, survival analysis takes two components into account: 1- the time to the event, and 2- a status variable that indicates whether an individual has been censored or not[23].

In survival analysis, the assumption is that all individuals can eventually experience the desired event, such as death or relapse. However, with advancements in treatment, some patients are now cured and do not encounter the event over time[24]. This results in two groups: the cured or long-term survivors, who exhibit similar survival rates to the general population, and the uncured or short-term survivors. Including cured patients in survival models increases censoring, leading to an overestimation of survival rates [25]. To address this issue, cure models have been introduced[26]. These models aim to explore factors influencing the cure rate as well as those affecting short-term survival[27]. In our study, we employed mixture and non-mixture cure models to examine the survival of patients with thyroid cancer. Additionally, we measured the factors influencing both survival rate and the cure probability of cure for thyroid cancer.

Materials and Methods

Data collection

In this historical cohort study, clinical information from 507 thyroid cancer patients was gathered from the Cancer Genome Atlas (https://www.cancer.gov/ccg/research/genome-sequencing/tcga) database. Additionally, 59 control samples were extracted from the TCGA database for comparison with cancer samples. The variables examined included gender, age, race, pathological stage, tumor multicenter status, primary thyroid gland neoplasm, primary neoplasm focal type, BRAF gene, radiation information genotype, and *TIMP3* expression. This study assessed two outcomes: survival time and cure status. Survival time was defined as the duration from diagnosis to death or the last follow-up time. Cure status represents the complete treatment of the disease, considered a latent variable determined by the model. All thyroid cancer patients whose information was recorded in TCGA database were included in the study. Patients for whom the time of diagnosis or expression of the *TIMP3* gene was unknown were excluded.

Data analysis

Following the description of the data, an independent sample t-test was employed to compare *TIMP3* expression between two groups: tumor-positive and normal tissue. The Kaplan-Meier curve was fitted to determine time to cure and cure fraction, and the log-rank test used for group comparisons. Intuitively, the cure fraction can be estimated by the difference between the smooth sequence of the Kaplan-Meier curve and the zero value on the survival probability axis.

Subsequently, Weibull Mixture Cure Model and Weibull Non-Mixture Cure Model were utilized to explore the association between *TIMP3* gene expression and survival time, as well as cure status. These models were applied in both simple and multiple settings, controlling for other factors. Odds of cure were quantified by the odds ratio (OR), while the hazard ratio (HR) indicated the risk of death. *TIMP3* expression was log2-transformed before all statistical analyses to mitigate extremely skewed gene expression distributions. Using the median of the data, the high and low expression of *TIMP3* was determined. Mixture cure models, commonly employed for long-lived data failure time modeling, pose the challenge of determining cure fraction and distribution parameters [28]. In the mixture cure model, the population is a mixture of cured and uncured patients and the model evaluates cure fraction and risk of death in uncured patients.

The second type of cure models are known as non-mixture cure model or bounded cumulative hazard model [29, 30]. This model assumes that the cumulative hazard is bounded from above in the presence of a cure fraction. The interest of this model lies in biological interpretation, so in the context of cancer, it can also be obtained by assuming that survival time is the result of latent processes. Finally, the best fit model was selected based on AIC criteria.

Software

All statistical analyses were conducted using the R language and environment for statistical computing (R version 4.3.1; R Foundation for Statistical Computing; www.r-project.org). The flexsurvcure package was utilized for computing both mixture and non-mixture cure models. Visualization of the data was performed using the ggplot2 package. Statistical significance at the level of 0.05 was

determined based on confidence intervals and p-value.

Results

In the study involving 507 participants, 136 (27%) were men, and 371 (73%) were women. Among the 59 control samples, 17 (29%) were men, and 42 (71%) were women. The average age of the patients was 47.93 ± 15.96 years (ranging from 15 to 89 years) and the average age of the individual was 47.06 ± 17.74 years (ranging from 15 to 81 years). According to the study criteria, 338 individuals (67%) had stage 1 disease, and 169 individuals (33%) had stage 2. In terms of racial distribution, 337 participants (66%) were white, 27 (5%) were of Asian, and 51 (11%) were African American. The race of 93 participants (18%) was not reported. Among the 507 participants, 16 individuals (3%) experienced the incident, and 270 individuals (53%) had unifocal glands, while 227 individuals (45%) had Multifocal glands (Table 1).

In individuals with tumor-positive tissue, the average *TIMP3* gene expression was 14.65 ± 1.79, while in those with normal tissue, it was 15.68 ± 0.72. The relationship between the two groups (tumor-positive and normal tissue) was found to be statistically significant ($p < 0.001$) (Table 2).

Based on the Figure 1, even after nearly 10 years of investigation, the survival rate of thyroid cancer patients does not reach zero, and the graph has plateaued since 2000 days. The estimated proportion of cured individuals is 78%. This evidence suggests the presence of cured individuals among the patients. Therefore, it is reasonable to employ cured models for this type of data.

In the study with 507 tumor-positive samples and 59 normal tissue samples, Figure 2 (c) displays the survival curve of thyroid cancer patients, considering gender. Furthermore, 137 male samples and 370 female samples are tumor-positive, and 17 male samples and 42 female samples have normal tissue. The log-rank test revealed no significant relationship between gender groups.

Figure 2 (b) illustrates the survival plot based on the presence or absence of a tumor. According to the graph, there was no significant difference between the two groups, with a p-value of 0.84. In Figure 2 (a), the survival plot is drawn based on *TIMP3* gene expression, categorizing gene expression data as high and low. The Kaplan-Meier curve showed a significant relationship between the two groups of high and low expression, with a p-value of 0.036, considering the convergence of gene expression data and comparison with the control sample.

A violin plot along with a box plot has been generated to illustrate the dispersion of *TIMP3* gene expression in the two groups: tumor-positive and normal tissue. This combined visualization depicts the level of gene expression and the degree of dispersion within each group, providing a comprehensive view of the distribution characteristics of the data (Figure 3).

In the evaluation of patient survival using both mixture

Table 1. Demographic, Clinical, and Laboratory Characteristics of Thyroid Cancer Patients

Variable		Count (%)		
		Male (N=136)	Female (N=371)	Total (N=507)
Race List	White	88 (64.7)	248 (66.8)	336 (66.27)
	Asian	6 (4.4)	21 (5.7)	27 (5.32)
	Black or African American	13 (9.6)	38 (10.2)	51(10.05)
	Not report	29(21.3)	64 (17.3)	93(18.34)
Vital Status	Alive	131 (96.3)	360 (97)	491 (96.84)
	Dead	5 (3.7)	11 (3)	16 (3.16)
Primary thyroid gland neoplasm	Right Lobe	49 (36)	165 (44.5)	214 (42.20)
	Left Lobe	43 (31.6)	131 (35.3)	174 (34.31)
	Bilateral	31 (22.8)	51 (13.7)	82(16.17)
	Isthmus	7 (5.1)	15 (4)	22 (4.34)
	Not report	6 (4.4)	9 (2.4)	15 (2.96)
Primary neoplasm focus type	Unifocal	57 (41.9)	213 (57.4)	270 (53.25)
	Multifocal	73 (53.7)	154 (41.5)	227 (44.77)
	Not report	6 (4.4)	4 (1.1)	10 (1.97)
Braf gene genotyping	Positive	8 (5.9)	10 (2.7)	18 (3.55)
	Negative	123 (90.4)	357 (96.2)	480 (94.67)
	Not report	5 (3.7)	4 (1.1)	9 (1.77)
Has radiations information	Yes	76 (55.9)	190 (51.2)	266 (54.46)
	No	60(44.1)	181(48.8)	241(45.54)
Pathologic stage	I	78(57.4)	260(70.1)	338(66.66)
	II	58(42.6)	111(29.9)	169(33.34)
TIMP3 expression	Low (Lower than median)	49(31.8)	105(68.2)	154(30.37)
	High (Higher than median)	87(24.6)	266(75.4)	353(69.62)

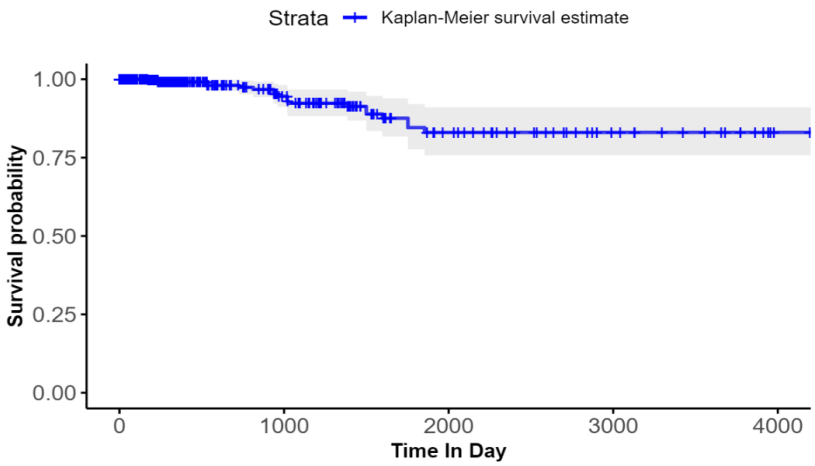

Figure 1. Estimating the Survival of Patients with Thyroid Cancer and Determining the Cure Time with the Kaplan-Meier Plot

Table 2. Comparison of Mean Values of *TIMP3* Expression between Tumor-Positive and Normal Tissue Groups

Variables	Patients with Tumor positive		Patients with Normal tissue		Test statistics	P-value
	Mean±SD	Number(n)	Mean±SD	Number(n)		
TIMP3 expression	1.79 ± 14.65	507	0.72 ± 15.68	59	4.32	<0.001

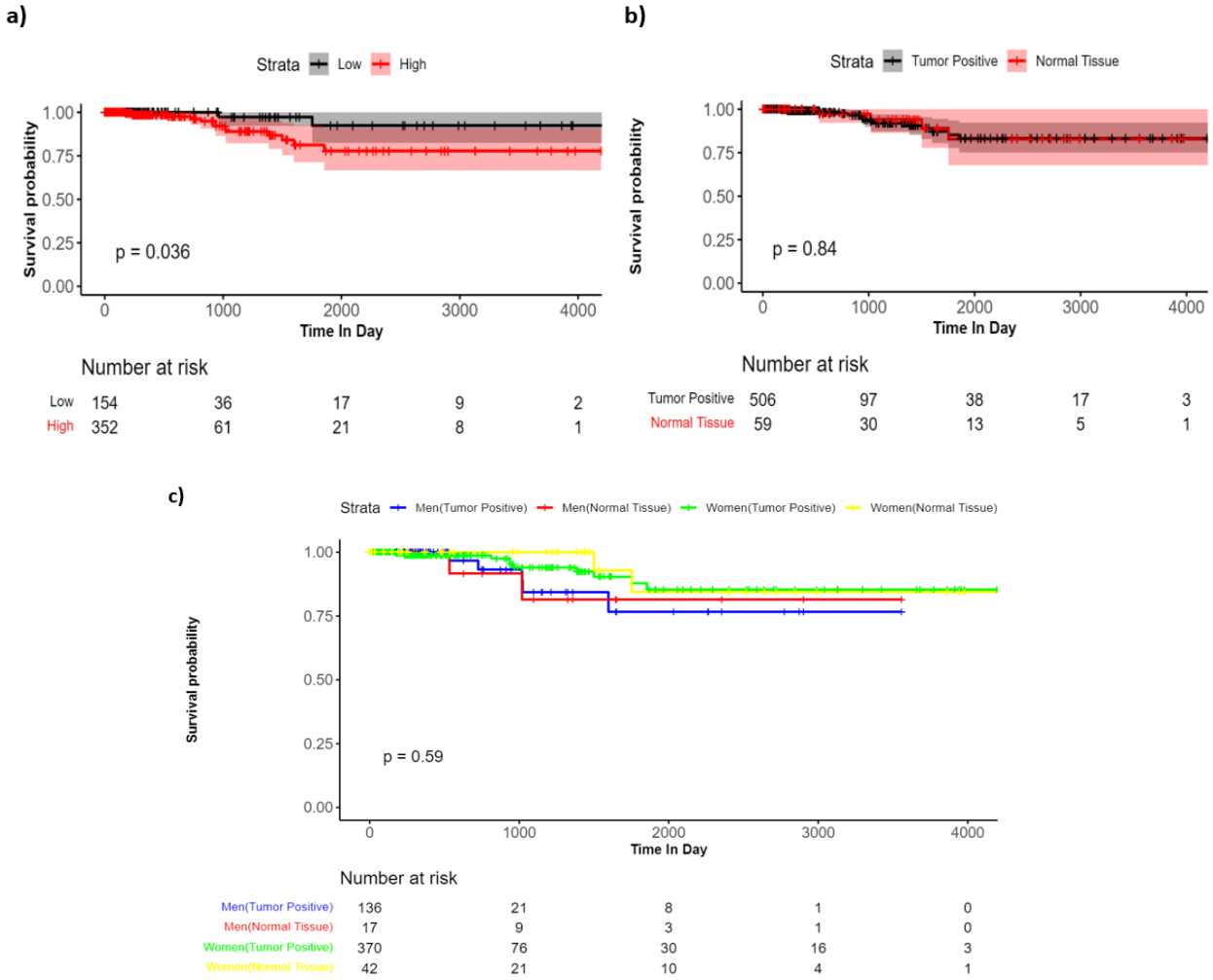

Figure 2. Survival Plot of a) thyroid cancer patients according to TIMP3 gene expression, b) patients with thyroid cancer according to the presence or absence of tumor and c) patients with thyroid cancer and control sample according to gender

Table 3. The Results of the Mixture Weibull Model in Univariate and Multiple Form

Variable		Simple Model Estimate (CI 95%)		Multiple Model Estimate (CI 95%)	
		Short-term survival	Long-term survival	Short-term survival	Long-term survival
Gender	Women	3.42 (1.14,10.18)	0.59 (0.21,1.72)	18.54 (2.05,164.02)	0.52 (0.14,1.88)
	Men†				
Stage	I	0.03 (0.01,0.11)	0.39 (0.1,1.43)	0.004 (0.0001,0.1)	0.13 (0.01,1.07)
	II†				
Right Lobe	Yes	0.91 (0.32,2.56)	1.28 (0.44,3.74)	0.51 (0.06,4.35)	0.44 (0.11,1.72)
	Other†				
Radiations	Yes†				
	No	0.35 (0.11,1.12)	1.13 (0.38,3.39)	0.11 (0.01,1.7)	1.21 (0.34,4.31)
Focus type	Unifocal†				
	Multifocal	3.74 (0.84,17.12)	1.15 (0.21,6.42)	4.81 (0.17,136)	0.35 (0.04,3.29)
TIMP3 expression		0.7 (0.41,1.17)	1.34 (0.75,2.39)	0.75 (0.43,4.18)	2.32 (1.04,5.21)

†, Reference; CI, confidence interval

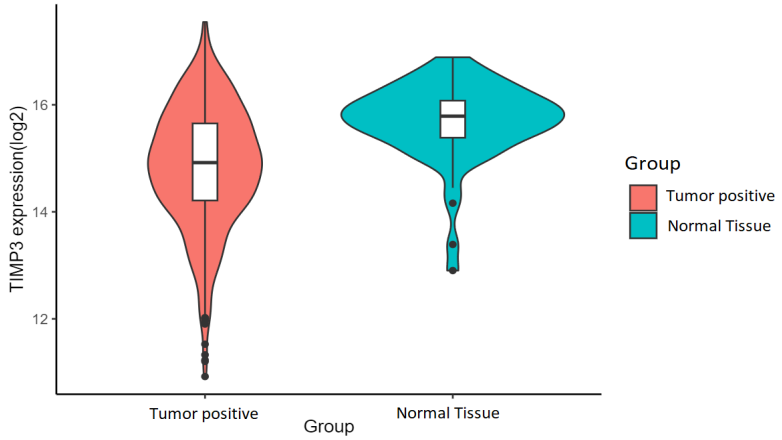

Figure 3. Violin Plot with Box Plot for *TIMP3* Expression in Patients with Tumor Positive vs Normal Tissue

and non-mixture Weibull models, the mixture cure model indicated a significantly lower risk hazard of death for women compared to men in the short term. Additionally, patients diagnosed at stage 2 disease had a lower risk than those at stage 1. However, in long-term survival, none of the variables showed a significant relationship with the odds of cure. In the multiple model for short-term survival, controlling for other factors yielded similar results to the univariate model. In the short-term , the risk of death was significantly risk for women than for men, and the hazard of death was lower for patients with stage 1 disease than for those with stage 2. In the long-term , when controlling

Table 4. The Results of the Non-Mixture Weibull Model in Univariate and Multiple Form

Variable		simple model Estimate (CI 95%)		multiple model Estimate (CI 95%)	
		Short-term survival	long-term survival	Short-term survival	long-term survival
Gender	Women	3.39 (1.12,10.18)	0.67 (0.21,2.12)	22.2 (1.88,259.82)	1.35 (0.29,6.36)
	Men†				
Stage	I	0.03 (0.01,0.12)	0.28 (0.07,1.11)	0.01 (0.001,0.08)	0.06 (0.004,0.07)
	II†				
Right Lobe	Yes	0.92 (0.33,2.59)	1.26 (0.41,3.94)	0.72 (0.11,4.71)	0.54 (0.09,3.32)
	Other†				
Radiations	Yes†				
	No	0.35 (0.11,1.09)	1.04 (0.32,3.42)	0.15 (0.02,1.01)	1.09 (0.28,4.35)
Focus type	Unifocal†				
	Multifocal	3.78 (0.16,17.12)	1.25 (0.21,7.24)	2.94 (0.19,45.6)	0.36 (0.04,3.06)
TIMP3 expression		0.7 (0.41,1.17)	1.28 (0.02,2.41)	0.96 (0.25,3.63)	2.01 (0.6,6.82)

†, Reference; CI, confidence interval

for other factors, *TIMP3* expression was associated with improved survival, so that, patients with higher *TIMP3* expression had higher odds of cure (Table 3).

Similarly, in the non-mixture cure model, the short-term results mirrored those of the mixture cure model, with a significantly lower probability of death for women than for men and a higher risk of early death for stage 2 patients compared to stage 1. However, for long-term survival, none of the variables demonstrated a significant relationship with the odds of cure. In accordance with the non-mixture cure model, akin to the mixture model, the short-term analysis revealed a significantly lower risk of death for women compared to men, and patients diagnosed with stage 2 disease had a higher risk than those with stage 1. However, in long-term survival, none of the variables exhibited a significant relationship with the odds of cure. In the multiple non-mixture cure model, controlling for other factors, the short-term analysis indicated a significantly lower risk of death for women than for men. Patients diagnosed with stage 2 disease more also less susceptible to short-term death than those diagnosed with stage 1. However, in the long-term, with the control of other factors, only the stage of the disease had a significant relationship with the cure probability. Specifically, the odds of cure in long-term in stage 2 patients was significantly lower than in those diagnosed at stage 1 (Table 4). Finally in comparison between two models, AIC criteria showed Weibull mixture cure model (AIC = 444.15) is more accurate and better fitting than the Weibull non-mixture cure model (AIC = 462.60).

Discussion

The present study provides an overview of modeling survival data considering the event of death in the presence of a cure fraction. In survival studies, if a portion of the population is immune to the event under consideration, cure models are employed. These models classify individuals into two groups: the sensitive group with short-term survival and the non-susceptible group with long-term survival. Those in the long-term survival group are considered immune to the event in question [31]. Cure models are categorized into mixture and non-mixture models, which account for both short-term and long-term effects. Notably, these models simplify to common survival models when there is no cure fraction.

In this study, the Weibull model was applied to evaluation of thyroid cancer survival using both the mixture and the non-mixture cure model. The dataset included information from 507 patients with thyroid cancer and 59 individuals with normal tissue who were followed up for 10 years.

Previous studies have indicated that the *TIMP3* gene plays a protective role against tumor growth by suppressing factors such as tumor growth, metastasis, angiogenesis, and apoptosis [21]. In this study, we specifically examined the impact of *TIMP3* gene expression on the survival of patients with thyroid cancer.

Both models revealed a significantly higher short-term mortality risk for women compared to men, and a reduced mortality risk was observed among stage 2 patients compared to those in stage 1.

Moreover, the results demonstrated a significant effect of between *TIMP3* gene expression and long-term survival when controlling for other factors related to thyroid cancer. Interestingly, the expression level of the *TIMP3* gene in individuals with tumor-positive tissue was found to be lower than in those with normal tissue [20].

The obtained results align with previous studies that have consistently demonstrated the protective effects of the *TIMP3* gene in thyroid cancer and other cancer types. In a 2011 animal study by Anania et al, increased *TIMP3* expression was found to diminish cancer cells' adhesive, migratory, and invasive capabilities, along with reducing tumorigenicity through decreased angiogenesis and macrophage infiltration [21]. Subsequently, in 2022, Mazzoni et al confirmed the association between *TIMP3* gene expression and papillary thyroid cancer in human data [22].

Research has also explored the correlation between *TIMP3* and other cancers. In a study by Chun-Wen Su et al, it was demonstrated that the overexpression of *TIMP3* gene reduces the migration and invasion abilities of oral cancer cells, inhibiting metastasis to lymph nodes in the body. Additionally, the findings indicated that the suppression of *TIMP3* by DNA methylation contributes to oral cancer metastasis [32].

The reduced levels of *TIMP3* in cancer may indeed result from genetic changes. In Su et al.'s study, patients with oral cancer carrying the rs9862TT genotype of the *TIMP3* polymorphism exhibited significantly higher *TIMP3* plasma levels compared to those with the CC genotype [33]. Dudea-Simon et al., in their research on cervical cancer patients, observed that decreased expression of MEG3 could serve as a warning signal in the transition from a malignant cervical lesion to invasive cancer. Furthermore, expression levels of SOX1, *TIMP3*, MALAT1, and MLH1 were identified as potential early markers in the diagnosis of cervical malignancy [34]. Gene expression data analysis has also revealed *TIMP3* down-regulation in thyroid cancer compared to healthy thyroid tissues, indicating a negative regulatory role in thyroid carcinogenesis. *TIMP3* attenuation has been observed in various cancers, including melanoma, choriocarcinoma, renal cell carcinoma, esophageal adenocarcinoma, as well as cancers affecting the kidney, brain, and other organs [35-37]. This study utilized data from thyroid cancer patients collected over a ten-year period from the TCGA website. However, a limitation is the relatively small sample size. Given the low mortality associated with this disease, increasing the number of available samples would enhance the validity and generalizability of the results.

In summary, the study highlights the prognostic significance of *TIMP3* gene expression in thyroid cancer, showing that higher *TIMP3* levels are linked to improved long-term survival and cure probability. This supports *TIMP3*'s role as a tumor suppressor, inhibiting growth, metastasis, and angiogenesis. Reduced *TIMP3* expression in tumors compared to normal tissue suggests its potential as a novel biomarker for prognosis and a therapeutic target. While the study is limited by a small sample size,

it underscores the need for further research to validate these findings and explore *TIMP3*'s mechanisms in thyroid cancer progression and treatment response. Ultimately, *TIMP3* could enhance risk assessment and inform targeted therapies for thyroid cancer patients.

Author Contribution Statement

Conceptualization: JY; Methodology: MMD,JY,AF; Software: MMD,JY,AF; Validation: JY; Formal analysis: MMD, JY,AF; Investigation: JY; Resources: JY,MMD; Data curation: MMD; Writing (original draft preparation): MMD,JY,AF,ZK; Writing (review and editing): MMD,JY,AF,ZK; Visualization: MMD; Supervision: JY; Project administration: JY; Funding acquisition: JY

Acknowledgements

Research Contribution

We hereby declare that this research is a part of an approved master's thesis of first author at Mazandaran university of medical sciences.

Ethical Approval and Consent to participate

The study protocol was following the Helsinki Declaration and was confirmed by the Ethics Committee of Mazandaran University of Medical Sciences (Approval Code: IR.MAZUMS.IMAMHOSPITAL.REC.1402.17176). The participants were informed about the research objectives and an informed consent form was obtained from the subjects before starting the survey.

Conflict of interests

The authors hereby affirm that the manuscript is original, that all statements asserted as facts are based on author's careful investigation and accuracy, that the manuscript has not been previously published in total or in part and has not been submitted or considered for publication in total or in part elsewhere. Each author acknowledges he/she has participated in the work substantively and is prepared to take public responsibility for the work and authors have no competing interest in the results of the article.

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