[InaBJ] M2020160 Manuscript Initial Check

4 messages

Secretariat of InaBJ <secretariatinabj@gmail.com> Mon, Jul 27, 2020 at 8:03 AM
To: Novalia Guntarno <novalia.guntarno@gmail.com>, anny_sr@fk.unair.ac.id

Dear Dr. Anny Setijo Rahaju,

Good day. Thank you for your submission of manuscript "**Role of VEGF and MMP-9 in T Stadium Bladder Urothelial Carcinoma**" on July 22, 2020. Your manuscript has been coded as M2020160, please note this code for your reference to communicate with us regarding this manuscript in the future.

Before your manuscript is sent to our reviewers, it has been initially checked. This manuscript needs some revisions. For a detailed list of corrections, please find it in the manuscript attached. Please **mark/highlighted the revised part of the manuscript**, so that editor will notice the changes. Herein we also attach an example on how to give a size marker for your figures.

Please send us an email of your corrected manuscript before **August 3, 2020** so that we can proceed with the peer-reviewing process. If you have any questions, do not hesitate to contact us.

Thank you. Please let us know if you have any questions. We wish you a nice day.

Best Regards,

Secretariat of The Indonesian Biomedical Journal
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2 attachments

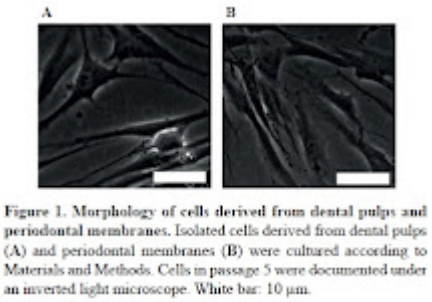

Figure 1. Morphology of cells derived from dental pulps and periodontal membranes. Isolated cells derived from dental pulps (A) and periodontal membranes (B) were cultured according to Materials and Methods. Cells in passage 5 were documented under an inverted light microscope. White bar: 10 μm.

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Novalia Guntarno <novalia.guntarno@gmail.com> Mon, Jul 27, 2020 at 8:25 AM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>
Cc: anny_sr@fk.unair.ac.id

Received, thank you.
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Novalia Guntarno <novalia.guntarno@gmail.com> Fri, Jul 31, 2020 at 9:09 AM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>
Cc: anny_sr@fk.unair.ac.id

Good morning, here i send the attachment of my revision, thank you

On Thu, 30 Jul 2020, 23:58 Novalia Guntarno, <novalia.guntarno@gmail.com> wrote:

> good night, this I send the revision of my article, thank you
> [Quoted text hidden]

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Novalia Guntarno <novalia.guntarno@gmail.com> Mon, Nov 16, 2020 at 6:32 AM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>
Cc: anny_sr@fk.unair.ac.id, Nila kurniasari <drnilakurniasari@gmail.com>

Good morning, is there any update of my article review? Thank you

On Mon, 2 Nov 2020, 11:44 Secretariat of InaBJ, <secretariatinabj@gmail.com> wrote:

> Dear dr. Novalia Guntarno,
>
> Good day. We sincerely apologize that currently we are still waiting for the review result from one of the peer-reviewer. We will soon contact you once the reviewer has sent their review result.
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> Thank you so much for your understanding. We wish you a nice day.
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> Best Regards,
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> On Mon, Nov 2, 2020 at 11:34 AM Novalia Guntarno <novalia.guntarno@gmail.com> wrote:
>
> > Good morning, is there any updates of my article review? Thank you very much
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> > On Tue, 27 Oct 2020, 06:05 Novalia Guntarno, <novalia.guntarno@gmail.com> wrote:
> >
> > > Good morning, is there any updates of my article review? Thank you very much
> > >
> > > On Fri, 9 Oct 2020, 10:00 Secretariat of InaBJ, <secretariatinabj@gmail.com> wrote:
> > >
> > > > Dear Authors,
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> > > > Good day. We would like to inform you that we are currently still waiting for the review result from one of the reviewers.
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> > > > Thank you for your understanding. We wish you a nice day.
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> > > > Best Regards,
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> > > > On Wed, Oct 7, 2020 at 6:38 AM Novalia Guntarno <novalia.guntarno@gmail.com> wrote:
> > > >
> > > > > Good morning, is there any updates of my article review? Thank you
> > > > > [Quoted text hidden]
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> > > > --
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[InaBJ] M2020160 Editor Decision - Resubmit for Review

2 messages

Secretariat of InaBJ <secretariatinabj@gmail.com> Thu, Nov 19, 2020 at 2:26 PM
To: Novalia Guntarno <novalia.guntarno@gmail.com>, anny_sr@fk.unair.ac.id

Dear Authors,

We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "**Role of VEGF and MMP-9 in T Stadium Bladder Urothelial Carcinoma**".

Our decision is to: **Resubmit for Review**.

This manuscript is interesting, however it needs major revision to improve the quality of the manuscript. We also found some grammatical and typographical errors, and also confusing sentences in the manuscript. Please read all the manuscripts and make sure that you revise the errors and proof-read them before you submit it back.

Find the file attached to see our reviewer's detailed comments. Please revise this manuscript according to reviewers' suggestions, and provide us a revised version of your manuscript and a response letter to reviewer before **December 7, 2020**.

Mark/highlighted the revised part of the manuscript, so that the editor will notice the changes. For an example on how to write a response letter, please find it here: http://www.jbc.org/content/suppl/2015/01/20/M114.610915.DC1/jbc.M114.610915-4.pdf.

When you are done, you can upload it in: https://inabj.org/index.php/ibj/author/submissionReview/1348, or simply send us an email.

Please let us know when you have received this email. If you have any questions, do not hesitate to contact us. Thank you for your attention. We wish you a nice day.

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Novalia Guntarno <novalia.guntarno@gmail.com> Fri, Nov 20, 2020 at 5:53 AM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>
Cc: anny_sr@fk.unair.ac.id

Received, thank you.
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M2020160 Review

1 message

Novalia Guntarno <novalia.guntarno@gmail.com> Sun, Dec 6, 2020 at 8:46 PM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>
Cc: anny_sr@fk.unair.ac.id, Nila kurniasari <drnilakurniasari@gmail.com>

> On Fri, 20 Nov 2020, 05:53 Novalia Guntarno, <novalia.guntarno@gmail.com> wrote:
> Received, thank you.
>
> On Thu, 19 Nov 2020, 14:26 Secretariat of InaBJ, <secretariatinabj@gmail.com> wrote:
> Dear Authors,
>
> We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "**Role of VEGF and MMP-9 in T Stadium Bladder Urothelial Carcinoma**".
>
> Our decision is to: **Resubmit for Review**.
>
> This manuscript is interesting, however it needs major revision to improve the quality of the manuscript. We also found some grammatical and typographical errors, and also confusing sentences in the manuscript. Please read all the manuscripts and make sure that you revise the errors and proof-read them before you submit it back.
>
> Find the file attached to see our reviewer's detailed comments. Please revise this manuscript according to reviewers' suggestions, and provide us a revised version of your manuscript and a response letter to reviewer before **December 7, 2020**.
>
> Mark/highlighted the revised part of the manuscript, so that the editor will notice the changes. For an example on how to write a response letter, please find it here: http://www.jbc.org/content/suppl/2015/01/20/M114.610915.DC1/jbc.M114.610915-4.pdf.
>
> When you are done, you can upload it in: https://inabj.org/index.php/ibj/author/submissionReview/1348, or simply send us an email.
>
> Please let us know when you have received this email. If you have any questions, do not hesitate to contact us. Thank you for your attention. We wish you a nice day.
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> Best Regards,
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~~Role of VEGF and~~ MMP-9 ~~in T Stage~~Promotes Invasion in Bladder Urothelial Carcinoma: it's Correlation with VEGF

Abstract

Background: Bladder cancer is the tenth-most common cancer in worldwide and the seventh-most common malignancy in men. The majority, 90–95%, of the bladder carcinomas are urothelial carcinomas. The depth of invasion plays important role in the prognostic and therapeutic factor in urothelial carcinomas. The expression of Vascular ~~e~~Endothelial G~~g~~rowth F~~f~~actor (VEGF) and Matrix metalloproteinase-9 (MMP-9) may associated with the progression of bladder carcinoma, such as depth of invasion. VEGF and its receptors play a central role in the process of angiogenesis which is an important role in tumor growth and progression of urothelial carcinoma. ~~Matrix metalloproteinases (~~MMPs~~)~~ have important roles in several cancer-supporting cellular processes, such as extracellular matrix (ECM) remodeling, angiogenesis, apoptosis, epithelial-to-mesenchymal transition and cell proliferation. In this study, the correlation between these two markers in urothelial bladder carcinoma invasion will be analyzed.

Methods: An analytical observational research with cross-sectional were conducted on 54 formalin fixed paraffin-embedded tissue from Radical Cystectomy (RC) which were diagnosed as bladder urothelial carcinoma at the Anatomical Pathology Laboratory of Dr. Soetomo General Hospital Surabaya during January 2010 - 31 August 2019. The samples of bladder urothelial carcinoma were divided based on the T stage and immunostained using VEGF and MMP-9 monoclonal antibodies. The difference of VEGF and MMP-9 expression in T stage of bladder urothelial carcinoma were ~~analysed~~analyzed using Kruskal-Wallis and Anova test, the correlation between VEGF and MMP-9 expression in various T stage of bladder urothelial carcinoma were ~~analysed~~analyzed using Spearman test.

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Result: This study showed no significant difference of VEGF expression among T stage of bladder urothelial carcinoma (p>0.05) but there was significant difference of MMP-9 expression in T stage of bladder urothelial carcinoma (p=0.043). There was a correlation between VEGF and MMP-9 in various T stage of bladder urothelial carcinoma ($r_{s=}$ 0.50, p=0.001).

Conclusion: The significant correlation of VEGF and MMP-9 expression in bladder urothelial carcinoma may prove the synergistically role of both proteins in tumor invasion by MMP-9 degradation extracellular matrix.

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Keywords: urothelial carcinoma, VEGF, MMP-9, T stage

Introduction

Bladder carcinoma is the ninth malignancy worldwide and seventh of the most common malignancy in men. Based on *Global Cancer Statistic* (GLOBOCAN) 2018, bladder carcinoma was the tenth malignancy with 549.000 cases and 200.000 cases of mortality rate (1).

Urothelial cell carcinoma is the most common histopathology subtype in bladder carcinoma, accounting for about 90% of all bladder malignancies (2). Determination of the degree of invasive tumor in bladder carcinoma is very important both for prognosis and the determination of therapy. T1 stage is stage of bladder carcinoma that has not yet penetrated to the muscular layer has a recurrence rate of 50 to 70% with good prognosis and the management of the treatment is provided by cystoscopic resection and intravesicular therap. Radical cystectomy can be performed in T1 stage with high risk condition such as tumor size >3 cm and multiple tumors. While T2, T3, T4 stage are stage of bladder carcinoma that has penetrated the muscular layer has a worse prognosis

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with combination therapy between cystectomy and cisplatin-based combination chemotherapy (3,4,5,6). Therefore, it is important to analyze the factors that play a role in bladder carcinoma invasion.

One of the most important factors in tumor growth and invasion is angiogenesis. Rapid tumor growth increases the need for oxygen and nutrients for metabolic processes. Tumor cells require the formation of new blood vessels to deliver oxygen and nutrients to tumor cells that are very proliferative (7). Vascular endothelial growth factor (VEGF) is one of the angiogenic factor that important to stimulates angiogenesis and tumor growth family of growth factors and the VEGF receptor forms a tumor angiogenesis signal pathway. The significance of the prognosis of VEGF expression has been studied in various types of cancer and several studies state that VEGF has an important role in bladder cancer progression and invasion (8,9, 10).

The basis of angiogenesis is the migration of endothelial cells into the surrounding loose tissue. Matrix metalloproteinases (MMPs) has complex roles including degradation of extracellular matrix, release of proangiogenic substances such as VEGF play a role in facilitating tumor angiogenesis. (11,12). Matrix Metalloproteinase-9 (MMP-9) is the type of MMP that is involves in the process of degradation of basement membrane and the formation of new blood vessels are characteristic of the progression in bladder carcinoma (12). Degradation of basement membrane is the key of tumor invasion which is supported by angiogenesis to support the oxygen and nutrient needs. Therefore, this study was conducted to analyze the role and the relationship between VEGF and MMP-9 in invasion (T stage) bladder urothelial carcinoma.

Methods

This study had been approved by the Health Research Ethic Committee of Dr. Soetomo General Hospital Surabaya. (Ethical Clearance 1851/KEPK/II/2020).

Research Design and Sample

An analytical observational research with cross-sectional approach were conducted on 54 formalin fixed paraffin-embedded tissue from Radical Cystectomy (RC) which were diagnosed as bladder urothelial carcinoma at the Anatomical Pathology Laboratory of Dr. Soetomo General Hospital Surabaya during January 2010 - 31 August 2019. The samples of bladder urothelial carcinoma were divided based on the T stage (T1, T2, T3, T4) (Table 1).

Immunohistochemistry Staining

The paraffin blocks of samples were cut io 4µm sections with Leica microtome into slides, deparaffinized three times with xylol for 5 minutes each, and rehydrated through graded alcohol (96%, 96%,90%,80%) for 2 minutes each. To reduce nonspecific staining due to peroxidased block, the slides were incubated in hydrogen peroxide for 10-15 minutes. Antigen retrieval was achieved by microwaves treatment in sodium citrate buffer (pH 6.0) for 45 minutes at 95°C, then applied background sniper (Biogear - Excell Block). The slides then were incubated with monoclonal antibodies for VEGF (C-1 - sc 7269 dilution 1:200; Santa Cruz Biotechnology) and MMP-9 (7-11C - sc 13520 dilution 1:200; Santa Cruz Biotechnology) overnight, and washed in phosphates buffer saline. Secondary antibody (Biogear Universal HRP Excell Stain System – Biogear, BDK-HES125) then applied for 15-20 minutes at room temperature followed DAB chromogen for 5-15 minutes. Slides then were counterstained with Meyer's hematoxylin and dehydrated with 95% alcohol.

Evaluation of Immunohistochemical Expression

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Is there any normal sample as control in this study?

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The expression of VEGF and MMP-9 was then evaluated using light microscopes Olympus CX41RF at 400x magnification and documented using Olympus DP2-BSW. The results of VEGF expression were interpreted according to the percentage and intensity of immunoreactive product in the cytoplasm of tumor cells. The percentage of tumor cell positively stained was determine by counting the tumor cell that had the highest immunoreactivity. Less than 10% was scored 0, more than 10% to 25% indicated score 1, more than 25% but less than 50% was scored 2 and more than 50% of staining was scored 3. The staining intensity were scored as 1,2,3 for weak, moderate and strong. Then the extend and intensity score were summed as total score, 0-2,3-4,5-6 were considered as negative, positive and strong positive (13). The results of MMP-9 expression were interpreted according to the percentage of immunoreactive product in the cytoplasm of tumor cells. When the tumor cells showed less than 5% indicated negative, more than 5% to 25% indicated low levels, more than 25% but less than 50% intermediate and more than 50 high levels of staining indicated high (14).

Statistical Analysis

All statistical analyses were calculated using SPSS v 25.0. The comparison of VEGF was tested using Mann Whitney U test and MMP-9 using Anova test. The correlation between VEGF and MMP-9 was analyzed using Spearman test with $p<0.05$.

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Result

VEGF Expression in Bladder Urothelial Carcinoma

Positive immunoreaction was observed as a brown color staining localized in the cell cytoplasm. There was no significance difference of VEGF expression was found in various T stage of bladder urothelial carcinoma $p=0.322$ ($p>0.05$) (Table 3). This study found that VEGF

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expression in the T1 stage samples had a final score that was evenly distributed from negative to strong positive expressions. Half of the samples in stages T2 and T3 were expressed with score 3 with VEGF and most of the samples in stages T4 were expressed with score 5-6.

MMP-9 Expression in Bladder Urothelial Carcinoma

Positive immunoreaction was observed as a brown color staining localized in the cell cytoplasm. There was a significance difference of MMP-9 expression in various T stage of bladder urothelial carcinoma p=0.043 (p<0.05) (Table 4). MMP-9 expression at T1 stage in this study were expressed score 3 in 50% cases. The data distribution of the MMP-9 expression group was normal, so it was followed by LSD test

Correlation between VEGF and MMP-9 in Bladder Urothelial Carcinoma

The correlation between VEGF and MMP-9 expression in bladder urothelial carcinoma was significant positive correlation r_s= 0.50; p=0.001 (p<0.05).

Discussion

This study found that VEGF expression in the 10 samples of T1 stage had final scores that were distributed from negative to strongly positive expressions. Half of the samples in stage T2 (7 samples) and T3 (7 samples) were strongly positive and most of the samples in stages T4 were strongly positive. Factors that can affect VEGF expression with T stage are VEGF receptors (VEGFR-1 and VEGFR-2), which are correlated with tumor invasion in bladder urothelial carcinoma (p = 0.01) (8). The most important interaction between VEGFR-2 and VEGF is the process of mitogenic, chemotactic, angiogenic and the increased permeability effects of VEGF. VEGF and VEGFR-2 bonds can trigger different signaling

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pathways, for example activation of the PLCγ – PKC – Raf kinase – MEK – mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3′ – kinase (PI3K) pathway that triggers changes in actin cytoskeleton and induces cell migration (8).

VEGF expressions in this study varied at each T stage. There are several factors that can regulate VEGF expression, including hypoxia, free radicals, pH imbalance, and nutrient deficiencies (15). To compensate the hypoxia and nutrient deficiencies and to escape from tumor necrosis, tumor cells express hypoxia-inducible factors (HIF), which will activate the transcription of over 40 genes, including VEGF (9). Suitable with the previous study, there was a positive correlation between HIF-1α and VEGF immunoreactivities (P<0.001) in urothelial carcinoma (16). VEGF and VEGF receptor transcription is directly activated by HIF-1α by binding hypoxia response element (HRE) and plays an important role during normal growth and tumor formation (17). In addition of hypoxia, conditions of pH imbalance and high temperature can also affect the level of VEGF transcription and VEGF expression through observation of nitric oxide (NO) donor sodium nitroprusside (SNP) and hydrogen peroxide (15). These factors may have more direct role in VEGF expression than their relation to T stage, and these factors have not been analyzed in this study.

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Commented [SoI21]: How did the authors explain the varied expression level of VEGF in this study and what is the correlation between the varied expression and the status of the samples which were a high-grade urothelial bladder carcinoma? Please explain whether the VEGF expression level is in a proportional manner with the T stage of the carcinoma and discuss further about that.

Commented [n22R21]: I've changed this paragraph because after I analyzed the data, there was no significance difference in between VEGF expression and tumor grade.

Commented [SoI23]: Revise this sentence

Commented [SoI24]: Explain in more detailed about this sentence, based on what data and theory? Give detailed parameters or characteristics of the T stage regarding the invasion ability.

Commented [SoI25]: What did the authors mean by tumor differentiation, because carcinoma cells do not differentiate.

MMP-9 is involved in several biological processes as a proteolytic that degrades extracellular matrix, separator between cells, separator of interactions between cells and extracellular matrix, division of proteins on cell surfaces, division of proteins in the extracellular environment (21). MMP-9 which is secreted by tumor cells is related to the ability to metastasize, this is because MMP-9 can degrade collagen types IV, V, VII and X. Type IV collagen is the main collagen supporting the basal membrane and is especially abundant in vascular endothelium in basal membrane, and acts as a barrier to invasion and metastasis. The high expression of MMP-9 causes degradation of extracellular matrix making it easier for tumor cells to invade (22,23).

The correlation between VEGF expression and MMP-9 expression in T-stage urothelial bladder carcinoma showed a significant result where the higher VEGF expression, the higher the MMP-9 expression in invasion bladder urothelial carcinoma. The results of this study are similar with previous study which stated a correlation between VEGF and MMP-9 in retinoblastoma tumor invasion. (24).

The limitation of this study was the small number of samples which originating from only one hospital leading to total sampling procedure.

Conclusion

MMP-9 expression is different on each depth of invasion in bladder urothelial carcinoma. The significant correlation of VEGF and MMP-9 expression in bladder urothelial carcinoma may The significant correlation of VEGF and MMP-9 expression in bladder urothelial carcinoma may prove

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Commented [SoI30]: Correlate the conclusion with the objective of this study.

the synergistically role of both proteins in tumor invasion by MMP-9 degradation extracellular matrix.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel R, Torre L, Jemal, A. Global cancer statistics 2018 : GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Ca Cancer J Clin. 2018; 68: 394–424.
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invasiveness of bladder cancer. Anticancer research. 2013; 33(6): 2381-2390.

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13. Al-Bassam, S. S. A., Kadhim H. S., Khashman, B. M. Possible association of vascular endothelial growth factor with grades of breast cancer. Int. J. Res. Pharm. Chem. 2014; 4:291-293.
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Table/Figures

Table 1. T Stages in Urothelial Bladder Carcinoma

T Stages	
T1	Tumor invades the submucosa or lamina propria
T2	Tumor invades muscle tissue
T3	Tumor extends beyond muscle into the perivesical fat
T4	Tumor invades the prostate, vagina, uterus, or bowel, or is fi xed to the abdominal wall, pelvic wall, or other organs

Table ~~1~~2. Clinicopathological Characteristic of Samples

Characteristics	N (%)
Age (years)*	
≤ 40	2 (3.7)
41-50	6 (11.10
51-60	14 (25.9)
61-70	26 (48.1)
71-80	5 (9.3)
≥ 81	1 (1.9)
Gender	
Male	50 (92.6)
Female	4 (7.4)
Tumor grade	
Low	7 (13)
High	47 (87)
T Stage	
T1	10 (18.5)
T2	14 (25.9)
T3	14 (25.9)
T4	16 (29.6)

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Table 3. VEGF Expression in Various T Stage Bladder Urothelial Carcinoma.

T Stage		T1	T2	T3	T4
Percentage	0 -10% (Score 0)	2 (20%)	2 (14.3%)	0	1 (6.2%)
	10 – 25% (Score 1)	2 (20%)	0	5 (35.7%)	1 (6.2%)
	25-50% (Score 2)	2 (20%)	1 (7.2%)	4 (28.6%)	3 (18.7%)
	> 50% (Score 3)	6 (60%)	11 (78.5%)	5 (35.7%)	11 (6.9%)
Intensity	Weak (Score 1)	5 (50%)	6 (42.8%)	6 (42.8%)	7 (43.7%)
	Moderate (Score 2)	4 (40%)	5 (35.7%)	5 (35.7%)	7 (43.7%)
	Strong (Score 3)	1 (10%)	3 (21.4%)	3 (21.4%)	2 (12.4%)
Total score	NEGATIVE (Score 0-2)	3 (30%)	2 (14.2%)	4 (28.5%)	2 (12.4%)
	POSITIVE (Score 3-4)	3 (30%)	5 (35.7%)	3 (21.4%)	7 (43.7%)
	STRONG POSITIVE (Score 5-6)	4 (40%)	7 (50%)	7 (50%)	7 (43.7%)
P	0.322				

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Table 43. MMP-9 expression in various T Stage Bladder Urothelial Carcinoma.

T Stage		T1	T2	T3	T4
Percentage	0 -5% (Score 0)	1 (10%)	0	0	0
	5 – 25% (Score 1)	1 (10%)	5 (35.7%)	1 (7.2%)	3 (6.2%)
	25-50% (Score 2)	3 (30%)	2 (14.2%)	5 (35.7%)	2 (12.5%)
	> 50% (Score 3)	5 (50%)	7 (50%)	8 (57.1%)	11 (6.9%)
Average		51.8	62.3	69.2	41.7
P	**0.043**				

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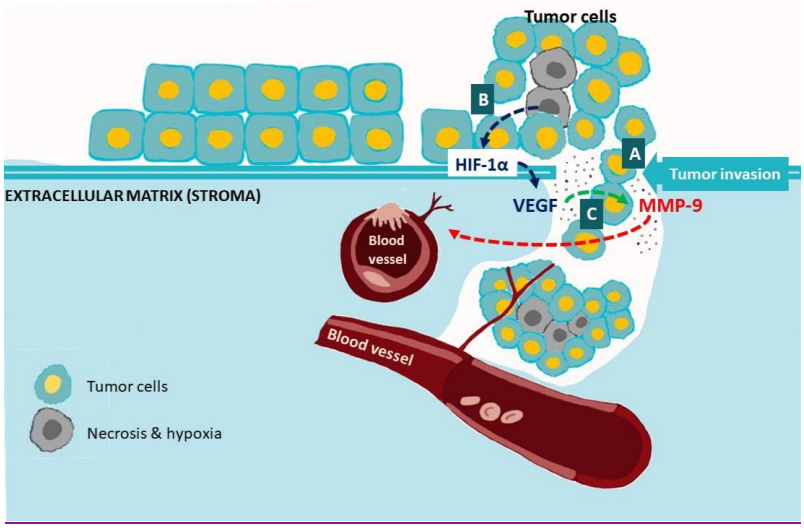
Table 5. Comparison of MMP-9 expression between two group

T Stage	*P* value
T1-T2	0.353
T1-T3	0.127
T1-T4	0.364
T2-T3	0.504
T2-T4	0.044
T3-T4	0.008

Figure 1. VEGF and MMP-9. A. The invasion process begins with the destruction of the basement membrane and extracellular matrix by the proteolytic enzyme MMP9 secreted by tumor cells., B. the uncontrolled proliferation of tumor cells results a lack of nutrient and oxygen supply to tumor cells. To prevent necrosis, tumor cells express HIF 1α. HIF 1α then triggers the release of VEGF which is the main mediator of tumor angiogenesis., C. at the same time, MMP9 secretion is also triggered by VEGF to support the angiogenesis process by degrading the extracellular matrix around blood vessels.



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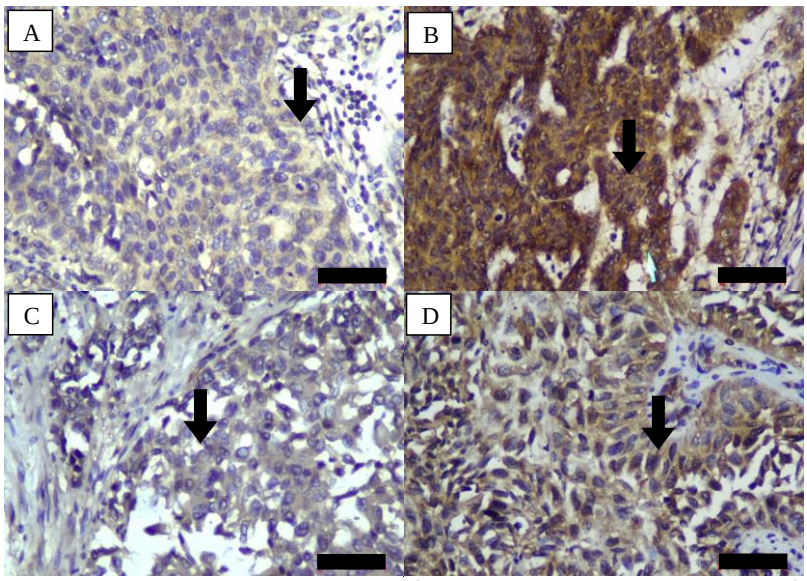
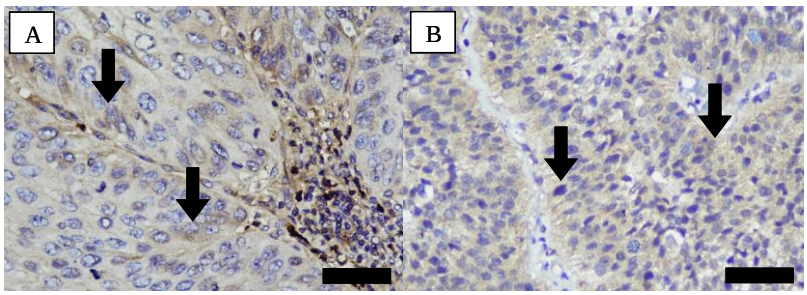


Figure 2. Immunohistochemical expression of VEGF expression in various T stage of bladder urothelial carcinoma, 400x magnification. T1 stage, 90% tumor cells were stained with weak intensity (A); T2 Stage, 65% tumor cell were stained with strong intensity (B); T3 stage, 70% tumor cells were stained with weak intensity (C); T4 stage, 95% tumor cells were stained with moderate intensity (D). Black bar : 5 µm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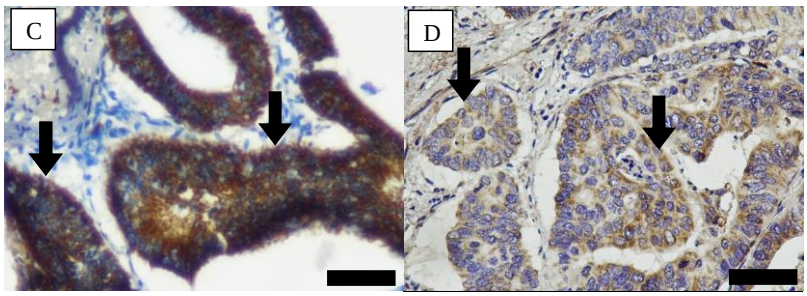

Figure 3. Immunohistochemical expression of MMP-9 expression in various T stage of bladder urothelial carcinoma, 400x magnification. T1 stage, 70% tumor cells were stained (A); T2 stage, 90% tumor cells were stained (B); T3 stage, 100% tumor cells were stained (C); T4 stage, 35% tumor cells were stained (D). Black bar : 5 μm

[InaBJ] M2020160 Editor Decision Round 2 - Revisions Required

1 message

Secretariat of InaBJ <secretariatinabj@gmail.com> Tue, Dec 22, 2020 at 2:53 PM
To: anny_sr@fk.unair.ac.id, Novalia Guntarno <novalia.guntarno@gmail.com>, drnilakurniasari@gmail.com

Dear Authors,

We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "**Role of VEGF and MMP-9 in T Stadium Bladder Urothelial Carcinoma**".

Our decision is to: **Revisions Required**.

Thank you for your revisions in the previous round of review, however the manuscript still needs some revision to improve the quality of the manuscript. Find the file attached to see our reviewer's detailed comments and corrections.

Please revise this manuscript according to reviewers' suggestions, and provide us a revised version of your manuscript and a response letter to reviewer before **January 8, 2021**. Mark/highlighted the revised part of the manuscript, so that the editor will notice the changes.

When you are done, you can upload it in: https://inabj.org/index.php/ibj/author/submissionReview/1348, or simply send us an email.

Please let us know when you have received this email. If you have any questions, do not hesitate to contact us. Thank you for your attention. We wish you a nice day.

Best Regards,

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Correlation of MMP-9 and VEGF in the Invasion State of Bladder Urothelial Carcinoma

~~**MMP-9 Promotes Invasion in Bladder Urothelial Carcinoma : it's Correlation with VEGFc**~~

Abstract

Background: Bladder cancer is the tenth-most common cancer in worldwide and the majority are urothelial carcinomas. The depth of invasion plays important role in the prognostic and therapeutic factor in urothelial carcinomas. The expression of Vascular Endothelial Growth Factor (VEGF) and Matrix metalloproteinase-9 (MMP-9) may associated with the progression of bladder carcinoma, such as depth of invasion. In this study, the correlation between these two markers in urothelial bladder carcinoma invasion will be analyzed.

Methods: An analytical observational research with cross-sectional were conducted on 54 formalin fixed paraffin-embedded tissue from Radical Cystectomy (RC) which were diagnosed as bladder urothelial carcinoma at the Anatomical Pathology Laboratory of Dr. Soetomo General Hospital Surabaya and divided based on the T stage were immunostained using VEGF and MMP-9 monoclonal antibodies. The difference of VEGF and MMP-9 expression in T stage of bladder urothelial carcinoma were analyzed using Kruskal-Wallis and Anova test, the correlation between VEGF and MMP-9 expression in various T stage of bladder urothelial carcinoma were analyzed using Spearman test.

Result: This study showed no significant difference of VEGF expression among T stage of bladder urothelial carcinoma (p>0.05) but there was significant difference of MMP-9 expression in T stage of bladder urothelial carcinoma (p=0.043). There was a correlation between VEGF and MMP-9 in various T stage of bladder urothelial carcinoma ($r_{s=}$ 0.50, p=0.001).

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Consider to delete common information.

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Conclusion: The significant correlation of VEGF and MMP-9 expression in bladder urothelial carcinoma may prove the synergistically role of both proteins in tumor invasion by MMP-9 degradation extracellular matrix.

Keywords: urothelial carcinoma, VEGF, MMP-9, T stage

Introduction

Bladder carcinoma is the ninth malignancy worldwide and seventh of the most common malignancy in men. Based on *Global Cancer Statistic* (GLOBOCAN) 2018, bladder carcinoma was the tenth malignancy with 549.000 cases and 200.000 cases of mortality rate (1).

Urothelial cell carcinoma is the most common histopathology subtype in bladder carcinoma, accounting for about 90% of all bladder malignancies (2). Determination of the degree of invasive tumor in bladder carcinoma is very important both for prognosis and the determination of therapy. T1 stage is stage of bladder carcinoma that has not yet penetrated to the muscular layer has a recurrence rate of 50 to 70% with good prognosis and the management of the treatment is provided by cystoscopy resection and intravesicular therapy Radical cystectomy can be performed in T1 stage with high risk condition such as tumor size >3 cm and multiple tumors. While T2, T3, T4 stage are stage of bladder carcinoma that has penetrated the muscular layer has a worse prognosis with combination therapy between cystectomy and cisplatin-based combination chemotherapy (3,4,5,6). Therefore, it is important to analyze the factors that play a role in bladder carcinoma invasion.

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One of the most important factors in tumor growth and invasion is angiogenesis. Rapid tumor growth increases the need for oxygen and nutrients for metabolic processes. Tumor cells require the formation of new blood vessels to deliver oxygen and nutrients to tumor cells that are very

proliferative (7). Vascular endothelial growth factor (VEGF) is one of the angiogenic factor that important to stimulates angiogenesis and tumor growth family of growth factors and the VEGF receptor forms a tumor angiogenesis signal pathway. The significance of the prognosis of VEGF expression has been studied in various types of cancer and several studies state that VEGF has an important role in bladder cancer progression and invasion (8,9, 10).

The basis of angiogenesis is the migration of endothelial cells into the surrounding loose tissue. Matrix metalloproteinases (MMPs) has complex roles including degradation of extracellular matrix, release of proangiogenic substances such as VEGF play a role in facilitating tumor angiogenesis. (11,12). Matrix Metalloproteinase-9 (MMP-9) is the type of MMP that is involves in the process of degradation of basement membrane and the formation of new blood vessels are characteristic of the progression in bladder carcinoma (12). Degradation of basement membrane is the key of tumor invasion which is supported by angiogenesis to support the oxygen and nutrient needs. Therefore, this study was conducted to analyze the role and the relationship between VEGF and MMP-9 in invasion (T stage) bladder urothelial carcinoma.

Methods

Research Design and Sample

This study had been approved by the Health Research Ethic Committee of Dr. Soetomo General Hospital Surabaya. (Ethical Clearance 1851/KEPK/II/2020).

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An analytical observational research with cross-sectional approach were conducted on 54 formalin fixed paraffin-embedded tissue from Radical Cystectomy (RC) which were diagnosed as

bladder urothelial carcinoma at the Anatomical Pathology Laboratory of Dr. Soetomo General Hospital Surabaya during January 2010 - 31 August 2019. The samples of bladder urothelial carcinoma were divided based on the T stage (T1, T2, T3, T4) (Table 1).

Immunohistochemistry Staining

The paraffin blocks of samples were cut io 4µm sections with Leica microtome into slides, deparaffinized three times with xylol for 5 minutes each, and rehydrated through graded alcohol (96%, 96%,90%,80%) for 2 minutes each. To reduce nonspecific staining due to peroxidased block, the slides were incubated in hydrogen peroxide for 10-15 minutes. Antigen retrieval was achieved by microwaves treatment in sodium citrate buffer (pH 6.0) for 45 minutes at 95°C, then applied background sniper (Biogear - Excell Block). The slides then were incubated with monoclonal antibodies for VEGF (C-1 - sc 7269 dilution 1:200; Santa Cruz Biotechnology) and MMP-9 (7-11C - sc 13520 dilution 1:200; Santa Cruz Biotechnology) overnight, and washed in phosphates buffer saline. Secondary antibody (Biogear Universal HRP Excell Stain System – Biogear, BDK-HES125) then applied for 15-20 minutes at room temperature followed DAB chromogen for 5-15 minutes. Slides then were counterstained with Meyer's hematoxylin and dehydrated with 95% alcohol.

Evaluation of Immunohistochemical Expression

The expression of VEGF and MMP-9 was then evaluated using light microscopes Olympus CX41RF at 400x magnification and documented using Olympus DP2-BSW. The results of VEGF expression were interpreted according to the percentage and intensity of immunoreactive product in the cytoplasm of tumor cells. The percentage of tumor cell positively stained was determine by counting the tumor cell that had the highest immunoreactivity. Less than 10% was scored 0, more

than 10% to 25% indicated score 1, more than 25% but less than 50% was scored 2 and more than 50% of staining was scored 3. The staining intensity were scored as 1,2,3 for weak, moderate and strong. Then the extend and intensity score were summed as total score, 0-2,3-4,5-6 were considered as negative, positive and strong positive (13). The results of MMP-9 expression were interpreted according to the percentage of immunoreactive product in the cytoplasm of tumor cells. When the tumor cells showed less than 5% indicated negative, more than 5% to 25% indicated low levels, more than 25% but less than 50% intermediate and more than 50 high levels of staining indicated high (14).

Statistical Analysis

All statistical analyses were calculated using SPSS v 25.0. The comparison of VEGF was tested using Mann Whitney U test and MMP-9 using Anova test. The correlation between VEGF and MMP-9 was analyzed using Spearman test with $p<0.05$.

Result

The average age of the sample in this study was 60.59 years. The youngest age at diagnosis was 34 years while the oldest was 81 years old. The distribution of samples based on age groups was divided into 5 groups with a span of 10 years. The highest number of samples was found in the age group 41 - 50 years with 13 samples (32.5%), followed by the 51 - 60 years' age group with 10 samples (25%) (Table 2). The gender distribution was 50 samples (92.59%) from male patients and 4 samples from female patients (Table 2). In this study, it was found that the most cases were stage T4 with 16 cases (29.62%), followed by stages T2 and T3 with the same number of 14 cases (25.92%) and stage T1 as many as 10 cases (18.51%) (Table 2).

VEGF Expression in Bladder Urothelial Carcinoma

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Positive immunoreaction was observed as a brown color staining localized in the cell cytoplasm. There was no significance difference of VEGF expression was found in various T stage of bladder urothelial carcinoma p=0.322 (p>0.05) (Table 3). This study found that VEGF expression in the T1 stage samples had a final score that was evenly distributed from negative to strong positive expressions. Half of the samples in stages T2 and T3 were expressed with score 3 with VEGF and most of the samples in stages T4 were expressed with score 5-6 (Figure 1).

MMP-9 Expression in Bladder Urothelial Carcinoma

Positive immunoreaction was observed as a brown color staining localized in the cell cytoplasm. There was a significance difference of MMP-9 expression in various T stage of bladder urothelial carcinoma p=0.043 (p<0.05) (Table 4). MMP-9 expression at T1 stage in this study were expressed score 3 in 50% cases (Figure 2). The data distribution of the MMP-9 expression group was normal, so it was followed by LSD test to find out which group had the most significant difference. The LSD test showed that the T3 and T4 groups were the groups that had the most significant difference with p = 0.008 and the T2 and T4 groups with p = 0.04. The other groups did not have a significant difference (table 5).

Correlation between VEGF and MMP-9 in Bladder Urothelial Carcinoma

The correlation between VEGF and MMP-9 expression in bladder urothelial carcinoma was significant positive correlation r_s= 0.50; p=0.001 (p<0.05).

Discussion

This study found that VEGF expression in the 10 samples of T1 stage had final scores that were distributed from negative to strongly positive expressions. Half of the samples in stage T2 (7 samples) and T3 (7 samples) were strongly positive and most of the samples in stages T4 were

strongly positive. Factors that can affect VEGF expression with T stage are VEGF receptors (VEGFR-1 and VEGFR-2), which are correlated with tumor invasion in bladder urothelial carcinoma (p = 0.01) (8). The most important interaction between VEGFR-2 and VEGF is the process of mitogenic, chemotactic, angiogenic and the increased permeability effects of VEGF. VEGF and VEGFR-2 bonds can trigger different signaling pathways, for example activation of the PLCγ – PKC – Raf kinase – MEK – mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3′ – kinase (PI3K) pathway that triggers changes in actin cytoskeleton and induces cell migration (8).

VEGF expressions in this study varied at each T stage. There are several factors that can regulate VEGF expression, including hypoxia, free radicals, pH imbalance, and nutrient deficiencies (15). To compensate the hypoxia and nutrient deficiencies and to escape from tumor necrosis, tumor cells express hypoxia-inducible factors (HIF), which will activate the transcription of over 40 genes, including VEGF (9). Suitable with the previous study, there was a positive correlation between HIF-1α and VEGF immunoreactivities (P<0.001) in urothelial carcinoma (16). VEGF and VEGF receptor transcription are directly activated by HIF-1α by binding hypoxia response element (HRE) and plays an important role during normal growth and tumor formation (17). In addition of hypoxia, conditions of pH imbalance and high temperature can also affect the level of VEGF transcription and VEGF expression through observation of nitric oxide (NO) donor sodium nitroprusside (SNP) and hydrogen peroxide (15). These factors may have more direct role in VEGF expression than their relation to T stage, and these factors have not been analyzed in this study.

The different expression of MMP-9 on each T stage indicate a difference in expression of MMP-9 on the depth of tumor cell invasion. This is in accordance with the previous research which

conducted research on MMP-9 expression in the progression of urothelial bladder carcinoma and obtained an increase in MMP-9 expression is in line with increased tumor invasion (18,19). Likewise, in the prior study which compared normal bladder tissue and bladder urothelial carcinoma, they found an increase in MMP-9 expression (up to 50%) in bladder urothelial carcinoma compared with normal bladder tissue (20).

MMP-9 is involved in several biological processes as a proteolytic that degrades extracellular matrix, separator between cells, separator of interactions between cells and extracellular matrix, division of proteins on cell surfaces, division of proteins in the extracellular environment (21). MMP-9 which is secreted by tumor cells is related to the ability to metastasize, this is because MMP-9 can degrade collagen types IV, V, VII and X. Type IV collagen is the main collagen supporting the basal membrane and is especially abundant in vascular endothelium in basal membrane, and acts as a barrier to invasion and metastasis. The high expression of MMP-9 causes degradation of extracellular matrix making it easier for tumor cells to invade (22,23).

The correlation between VEGF expression and MMP-9 expression in T-stage urothelial bladder carcinoma showed a significant result where the higher VEGF expression, the higher the MMP-9 expression in invasion bladder urothelial carcinoma (Figure 3). The results of this study are similar with previous study which stated a correlation between VEGF and MMP-9 in retinoblastoma tumor invasion (24). The basis of angiogenesis is the migration of endothelial cells into the surrounding loose tissue. MMP-9 has complex roles including remodelling of extracellular matrix, and is activated by angiogenic factors, one of which is VEGF. Conversely, MMP-9 also promotes endothelial cell migration and triggers angiogenic switches (a balance effect between proangiogenic and anti-angiogenic) by releasing VEGF during the carcinogenesis process (11,25).

The limitation of this study was the small number of samples which originating from only one hospital thus making us use total sampling procedure. ~~leading to total sampling procedure~~.

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Conclusion

MMP-9 expression is different on each depth of invasion in bladder urothelial carcinoma. The significant correlation of VEGF and MMP-9 expression in bladder urothelial carcinoma may prove the synergistically role of both proteins in tumor invasion by MMP-9 degradation extracellular matrix.

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Table/Figures

Table 1. T Stages in Urothelial Bladder Carcinoma (3)

T Stages	
T1	Tumor invades the submucosa or lamina propria
T2	Tumor invades muscle tissue
T3	Tumor extends beyond muscle into the perivesical fat
T4	Tumor invades the prostate, vagina, uterus, or bowel, or is fi xed to the abdominal wall, pelvic wall, or other organs

Table 2. Clinicopathological Characteristic of Samples

Characteristics	N (%)
Age (years)*	
≤ 40	2 (3.7)
41-50	6 (11.10
51-60	14 (25.9)
61-70	26 (48.1)
71-80	5 (9.3)
≥ 81	1 (1.9)
Gender	
Male	50 (92.6)
Female	4 (7.4)
Tumor grade	
Low	7 (13)
High	47 (87)
T Stage	
T1	10 (18.5)
T2	14 (25.9)
T3	14 (25.9)
T4	16 (29.6)

Commented [19]: References?

Table 3. VEGF Expression in Various T Stage Bladder Urothelial Carcinoma.

T Stage		T1	T2	T3	T4
Percentage	0 -10% (Score 0)	2 (20%)	2 (14.3%)	0	1 (6.2%)
	10 – 25% (Score 1)	2 (20%)	0	5 (35.7%)	1 (6.2%)
	25-50% (Score 2)	2 (20%)	1 (7.2%)	4 (28.6%)	3 (18.7%)
	> 50% (Score 3)	6 (60%)	11 (78.5%)	5 (35.7%)	11 (6.9%)
Intensity	Weak (Score 1)	5 (50%)	6 (42.8%)	6 (42.8%)	7 (43.7%)
	Moderate (Score 2)	4 (40%)	5 (35.7%)	5 (35.7%)	7 (43.7%)
	Strong (Score 3)	1 (10%)	3 (21.4%)	3 (21.4%)	2 (12.4%)
Total score	Negative (Score 0-2)	3 (30%)	2 (14.2%)	4 (28.5%)	2 (12.4%)
	Positive (Score 3-4)	3 (30%)	5 (35.7%)	3 (21.4%)	7 (43.7%)
	Strong positive (Score 5-6)	4 (40%)	7 (50%)	7 (50%)	7 (43.7%)
***P* (total score)**	0.322				

Kruskal-Wallis test

Commented [110]: p-value for which variable?? Please also write a note under the table to show what statistical test did you use.

Table 4. MMP-9 expression in various T Stage Bladder Urothelial Carcinoma.

T Stage		T1	T2	T3	T4
Percentage	0 -5% (Score 0)	1 (10%)	0	0	0
	5 – 25% (Score 1)	1 (10%)	5 (35.7%)	1 (7.2%)	3 (6.2%)
	25-50% (Score 2)	3 (30%)	2 (14.2%)	5 (35.7%)	2 (12.5%)
	> 50% (Score 3)	5 (50%)	7 (50%)	8 (57.1%)	11 (6.9%)
Average		51.8	62.3	69.2	41.7
P	**0.043**				

Anova test

Commented [111]: Please also write a note under the table to show what statistical test did you use.

Table 5. Comparison of MMP-9 expression between two group

T Stage	*P* value
T1-T2	0.353
T1-T3	0.127
T1-T4	0.364
T2-T3	0.504
T2-T4	0.044
T3-T4	0.008

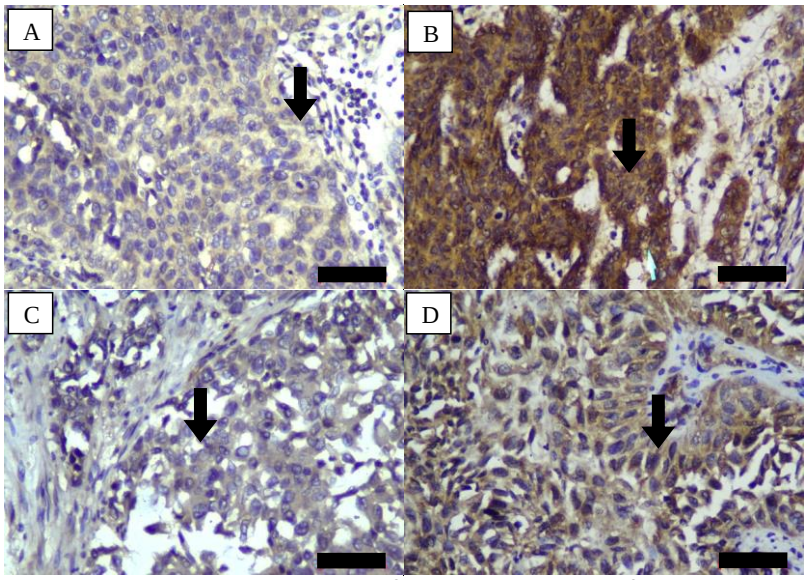

Figure 1. Immunohistochemical expression of VEGFin various T stage of bladder urothelial carcinoma~~, 400x magnification~~. T1 stage, 90% tumor cells were stained with weak intensity (A); T2 Stage, 65% tumor cell were stained with strong intensity (B); T3 stage, 70% tumor cells were stained with weak intensity (C); T4 stage, 95% tumor cells were stained with moderate intensity (D). Black bar : 5 µm

Commented [RIJ12]: Please cite this data in the text (Results section) and described the interpretation of this data

Commented [113]: Information about the magnification can be mentioned in the Methods.

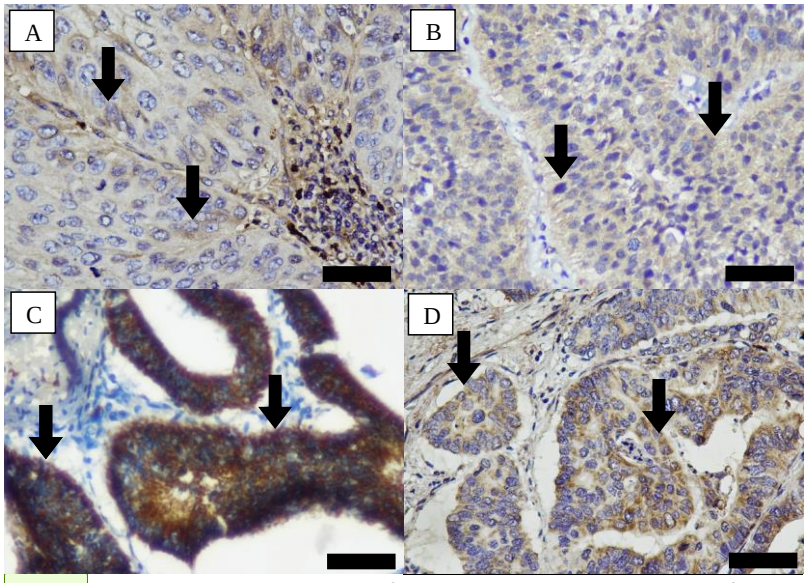

Figure 2. Immunohistochemical expression of MMP-9 expression in various T stage of bladder urothelial carcinoma. T1 stage, 70% tumor cells were stained (A); T2 stage, 90% tumor cells were stained (B); T3 stage, 100% tumor cells were stained (C); T4 stage, 35% tumor cells were stained (D). Black bar : 5 µm

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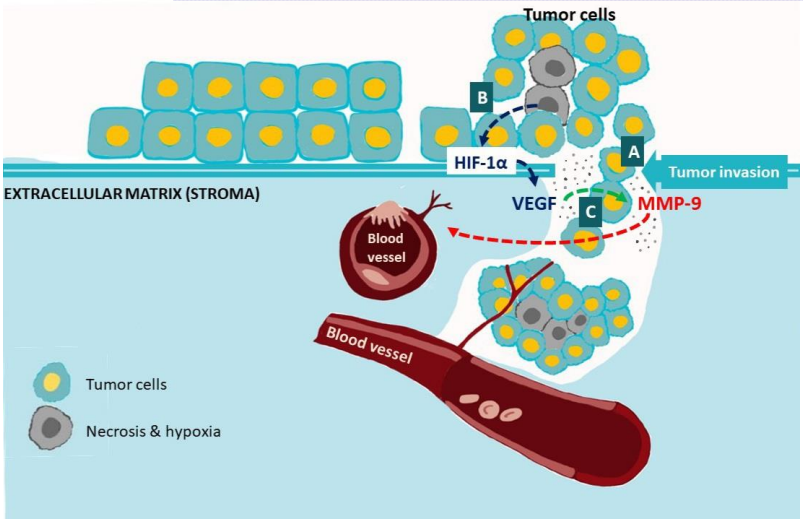
Figure 3. VEGF and MMP-9. A. The invasion process begins with the destruction of the basement membrane and extracellular matrix by the proteolytic enzyme MMP9 secreted by tumor cells., B. the uncontrolled proliferation of tumor cells results a lack of nutrient and oxygen supply to tumor cells. To prevent necrosis, tumor cells express HIF 1α. HIF 1α then triggers the release of VEGF which is the main mediator of tumor angiogenesis., C. at the same time, MMP9 secretion is also triggered by VEGF to support the angiogenesis process by degrading the extracellular matrix around blood vessels.



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