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Submission date: 21-Nov-2022 04:54PM (UTC+0800)
Submission ID: 1960180732
File name: Laryngeal_Squamous_Cell_Carcinoma_with_Cervical_Nodal_Status.pdf (1.89M)
Word count: 5742
Character count: 33124

ISSN-0973-9122 (Print) ● ISSN-0973-9130 (Electronic)

Volume 15 / Number 4 / October-December 2021

Indian Journal of Forensic Medicine & Toxicology

Website: www.ijfmt.com

Official Organ of Indian Association of Medico-Legal Experts (Regd.)

Indian Journal of Forensic Medicine & Toxicology

Print-ISSN:0973-9122 Electronic - ISSn: 0973-9130

Website: www.ijfmt.com

Editor

Dr. R.K. Sharma
Institute of Medico-legal Publications
Logix Office Tower, Unit No. 1704, Logix City Centre Mall, Sector- 32, Noida - 201 301 (Uttar Pradesh)

Printed, published and owned by

Dr. R.K. Sharma
Institute of Medico-legal Publications
Logix Office Tower, Unit No. 1704, Logix City Centre Mall, Sector- 32, Noida - 201 301 (Uttar Pradesh)

Published at

Institute of Medico-legal Publications
Logix Office Tower, Unit No. 1704, Logix City Centre Mall, Sector- 32, Noida - 201 301 (Uttar Pradesh)

"Indian Journal of Forensic Medicine & Toxicology" is peer reviewed quarterly journal. It deals with Forensic Medicine, Forensic Science, Toxicology, DNA fingerprinting, sexual medicine and environment medicine. It has been assigned International standard serial No. p-0973-9122 and e- 0973-9130. The Journal has been assigned RNI No. DELENG/2008/21789. The journal is indexed with Index Copernicus (Poland) and is covered by EMBASE (Excerpta Medica Database). The journal is also abstracted in Chemical Abstracts (CAS) database (USA. The journal is also covered by EBSCO (USA) database. The Journal is now part of UGC, DST and CSIR Consortia. It is now offical publication of Indian Association of Medico-Legal Experts (Regd.).

Correlation of HIF-1α, CXCR4 and MMP13 Expression in Laryngeal Squamous Cell Carcinoma with Cervical Nodal Status

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Abstract

Background: Laryngeal carcinoma is the most common malignancy of the upper respiratory tract, more than 98% are squamous cell carcinoma (SCC). New method is necessary for identifying and predicting nodal metastasis in laryngeal SCC. Overproliferating tumor cells will induce hypoxia and release HIF-1α, which in turn will upregulate CXCR4. CXCR4 then will induce MMP13, a protein that degrade extracellular matrix (ECM), thus promoting metastatic process.

Methods: A cross sectional study, using 30 samples of laryngeal SCC, divided into two groups: based on cervical lymph node status. All samples were stained immunohistochemically against HIF-1α, CXCR4 and MMP13 antibody. The expressions were evaluated using immunoreactive score (IRS).

Result: There were significant difference among HIF1α, CXCR4, and MMP13 in laryngeal SCC with positive nodal metastasis group compared to negative nodal metastasis group ($p<0,05$). There was no significant correlation between HIF-1α expression and CXCR4 expression ($p = 0,403$) ($p>0,05$). There were significant correlation between HIF-1α and MMP13 expression ($r_s = 0,499$), and between CXCR4 and MMP13 expression ($r_s = 0,409$). **Conclusion:** There were significant differences in HIF-1α, CXCR4 and MMP13 immunoexpression in laryngeal SCC with cervical nodal metastasis compared to laryngeal SCC without cervical nodal metastasis. There was a positive correlation between HIF-1α and MMP13 expression, positive correlation between CXCR4 and MMP13 expression, but there was no correlation between HIF-1α and CXCR4 expression.

Keywords: *Laryngeal carcinoma, lymph node metastasis, HIF-1α, CXCR4, MMP13*

Background

Laryngeal carcinoma is the most common malignancy of the upper respiratory tract. Globocan estimates that there will be 177,422 new cases and 94,771 deaths due to laryngeal carcinoma worldwide in 2018.[1] More than 98% of laryngeal malignancies are well differentiated SCC.[2]

Prognosis of laryngeal carcinoma depends greatly by tumor stadium. Laryngeal carcinoma confined to glottis has good 5 years survival rate (YSR), up to 85-95%, while presence of nodal metastasis will reduce 5 YSR significantly to 50%. The important significant single prognostic indicator of laryngeal carcinoma is the cervical nodal status.[3,4]

Identification of nodal metastasis in laryngeal carcinoma patients is not always easy. Probability of nodal metastasis without palpable nodal enlargement is about 13,7-37%, and better identification methods is indispensable.[5]

In SCC there will be changes in microenvironment of the tumor caused by proliferation of tumor cells. Up to certain tumor volume, blood and nutrients supply become inadequate and lead to tissue hypoxia. Hypoxia will induce HIF-1α, which is a major regulator of cellular response to changes in oxygen concentration supporting the adaptation of tumor cells to hypoxia in an oxygen-deficient tumor microenvirontment.[6] Overexpression of HIF-1α which in turn upregulates CXCR4.[7]

Chemokine is a family of chemotactic that modulate cell movement and positioning, and act by coupling to G-protein coupled receptor. CXCR4 is a unique chemokine receptor because it has exclusive interaction with its ligand, CXCL12. The binding of CXCR4 and CXCL12 will initiates various downstream signaling pathways, resulting in various responses such as rising intracellular calcium, genetic transcription, cell proliferation, migration, adhesion, and invasion. CXCR4 is a critical tumor marker with a proven role in cancer progressivity and metastasis, but its role in laryngeal cancer is not yet well studied.[8,9]

Infiltration of cancerous cell to the surrounding tissue is an important behavior in cancer progressivity. Proteolytic enzymes such as matrix metalloproteases (MMPs) contribute to tumor expansion by degrading extracellular matrix (ECM) components. Some studies showed expression of MMP in SCC. MMP13 has a vital role in MMP activation, and it's also expressed in some head and neck SCC.[10,11] Meanwhile, its role in nodal metastasis is not yet well known.

This study aims to analyse the expression of HIF-1α, CXCR4, and MMP13 in laryngeal SCC to unveil the roles of those markers in nodal metastasis process, which is expected to be an invaluable prognostic biomarker.

Material and Methods

This is an observational analytic study with cross-sectional approach. The population consists of all laryngeal SCC tissues with and without cervical nodal metastasis in paraffin blocks, archived in Anatomical Pathology Laboratory of Dr. Soetomo General Academic Hospital in Surabaya, during 2013 – 2016 period. The samples are 30 laryngeal SCC tissues in paraffin blocks from laryngectomy specimens, taken by random sampling methods.

In this study, immunoexpressions of these three proteins were evaluated by immunohistochemistry examination. We used monoclonal antibody against HIF-1α (NB100-131, Novusbio) with 1:100 dilution, monoclonal antibody against CXCR4 (sc-53534, Santa Cruz Biotechnology, Inc., Texas, USA) with 1:50 dilution, and monoclonal antibody against MMP13 (sc-101564, Santa Cruz Biotechnology, Inc., Texas, USA) with 1:50 dilution. All antigen retrievals were done using decloaking chamber (heat-induced epitope retrieval) with DIVA solution in 110° C for 30 minutes.

HIF-1α was positive if expressed in nucleus or cytoplasm of tumor cells. CXCR4 was positive if expressed in the nucleus, cell membrane or cytoplasm. MMP13 was positive if expressed in cytoplasm of tumor cells.

HIF-1α, CXCR4, and MMP13 immunohistochemical staining were assessed using semiquantitative Immunoreactivity Score (IRS) refferancing to Remmele and Stegner. To determine the IRS, the percentage of positive tumor cells was classified into five grade (no positive cells = 0, <10% of positive cells = 1, 10-50% positive cells = 2, 51-80% positive cells = 3, and > 80% positive cells = 4). Then the grade of positive cells percentage is multiplied by the grade of staining intensity which is divided into 4 grades (no staining = 0, weak staining = 1, moderate staining = 2, and strong staining = 3). The final IRS score ranges from 0-12.[12] The assessment was performed by two pathologists.

All datas were analyzed statistically using SPSS program. Analysis of expression differences of HIF-1α, CXCR4, and MMP13 was done with Mann-Whitney test. Analysis of correlation was done with Spearman correlation test. Statistical result is significant if $p < 0,05$.

Result

Mean age of all 30 patients was 55,9±8,36 years

old. The youngest was 49 years old and the oldest was 75. Patients were grouped into 4 groups with 10 years interval. As many as 53,33% patients were in the 51-60 years old group. Most patients were male. Out of 30 cases, there were only 2 female patients.

As many as 80% or 24 out of 30 cases of laryngeal SCC in this study were well differentiated. Four cases (12,33%) were moderately differentiated, and only 2 cases (6,67%) were poorly differentiated. Only 1 case (3,33%) was diagnosed in early stage (T1). Most cases, 43,33% or 13 cases of these cases were resected in stadium T3. There were 15 cases with cervical nodal metastasis; 8 cases (26,67%) in stadium N1 (metastasis to one ipsilateral lymph node with diameter ≤ 3 cm), and 7 cases with stadium N2.

Statistical analysis with Mann-Whitney showed that there was a significant difference among median scores of HIF1α, CXCR4 and MMP13 expression in laryngeal SCC without nodal metastasis group compared to laryngeal SCC with nodal metastasis group ($p<0,05$), as depicted in table 5.2.

In this study, Spearman correlation test showed no significant correlation between HIF-1α and CXCR4 expression, with $p = 0,403$ ($p>0,05$). Meanwhile, the test showed positive correlation between HIF-1α and MMP13, and between CXCR4 and MMP13 with $r_s = 0,499$ and $r_s = 0,409$ ($p < 0,05$), respectively.

Table 1: Sample Characteristics (n=30)

Parameter		Frequency	Percentage (%)
Age	£50	8	26,67
	51-60	16	53,33
	61-70	2	6,67
	>70	4	13,33
Sex	Male	28	93,33
	Female	2	6,67
Tumor Differentiation	Well	24	80
	Moderate	4	13,33
	Poor	2	6,67
Stadium T	1	1	3,33
	2	9	30
	3	13	43,33
	4a	7	23,33
	4b	0	0
Stadium N	0	15	50
	1	8	26,67
	2a	1	3,33
	2b	4	13,33
	2c	2	6,67
	3	0	0

Table 2: HIF1α, CXCR4 and MMP13 Expression in Laryngeal SCC with and without Nodal Metastasis

Stadium	n	HIF1a Expression					Mann Whitney P
		`x	SD	Median	Min	Max	
Without nodal metastasis	15	1	1,41	0	0	4	0,000*
With nodal metastasis	15	4,47	2,03	4	2	9	

Stadium	n	CXCR4 Expression					Mann Whitney p
		`x	SD	Median	Min	Max	
Without nodal metastasis	15	6,6	3,11	6	3	12	0,023*
With nodal metastasis	15	9,4	2,99	9	4	12	
Stadium	**n**	**MMP13 Expression**					**Mann Whitney**
		`x	**SD**	**Median**	**Min**	**Max**	**P**
Without nodal metastasis	15	1	0,85	1	0	3	0,000*
With nodal metastasis	15	2,8	1,26	2	0	4	

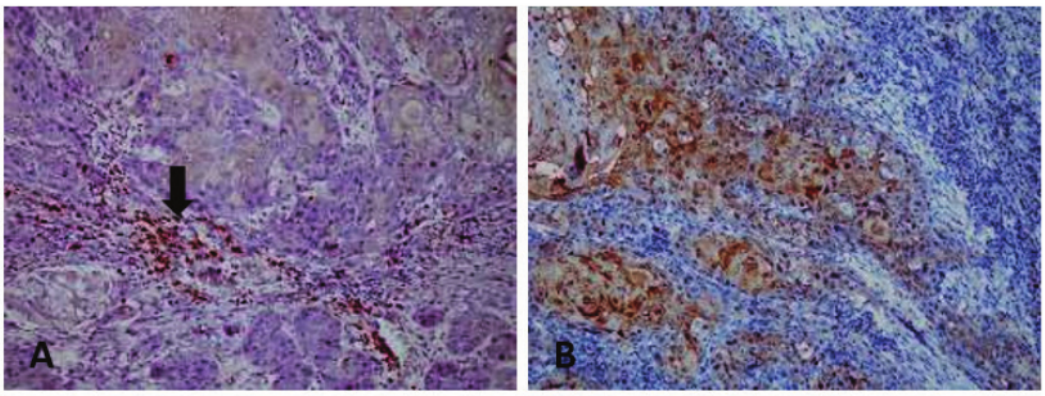

Figure 1. HIF1α expression, stained positively in cytoplasm of tumor cells. A, with weak intensity. Internal positive control was inflammatory cells (arrow) (HE, 200x). B, with strong intensity (HE, 200x).

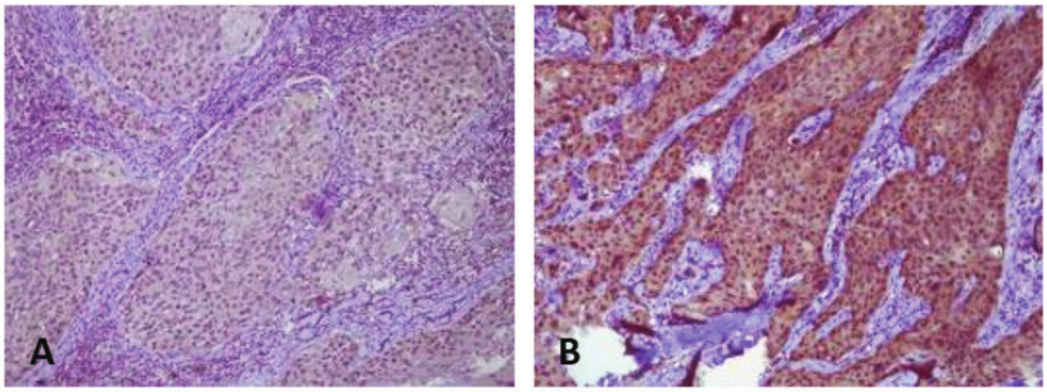

Figure 2. CXCR4 expression, stained positively in cytoplasm and nucleus of tumor cells. A, with weak intensity (HE, 200x). B, with strong intensity (HE, 200x).

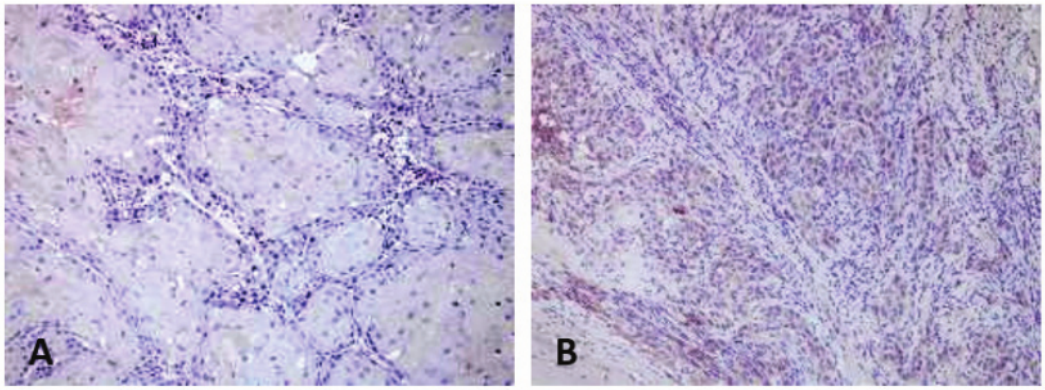

Figure 3. MMP13 expression, stained positively in cytoplasm of tumor cells. A, with weak intensity (HE, 200x). B, with moderate intensity (HE, 200x).

Discussion

In this study, statistical analysis showed that there was a significant difference in HIF-1α expression in laryngeal SCC with nodal metastasis compared to laryngeal SCC without nodal metastasis (p<0,05). HIF-1α was expressed stronger in metastasis group. This finding is in concordance with previous studies, such as studies by Wu et al and Popov et al who studied HIF-1α expression in laryngeal SCC.[13,14] and also in oral SCC.[15] Zhou et al in a meta-analysis study showed that HIF-1α associated with not only lymph node metastasis, but also tumor size, tumor stage and overall survival.[16]

HIF-1 is an important factor regulating cell adaptation to oxygen deprivation. In a normoxic condition, the proline and lysine residues of HIF-1α subunits are hydroxylated by oxygen-dependent prolyl-4-hydroxylases (PHDs). Von Hippel-Lindau E3 ubiquitin ligase binds to hydroxylated HIF-1α and acts as a substrate recognition component of E3 ubiquitin ligase complex, which leads to proteasomal degradation of HIF protein. In solid tumors like laryngeal SCC, hypoxia is commonly found since there is a higher proliferation rate and intratumoral oxygen will be rapidly depleted. In hypoxic condition, the activity of PHDs and FIHs are suppressed, and HIF-1α translocates to the nucleus to bind with HIF-1β. The heterodimeric HIF-1α with HIF-

1β with the help of coactivators such as cyclic adenosine monophosphate response element-binding protein (CBP) and acetyltransferase (p300), then bind to the target gene hypoxia response element (HRE), resulting in their transcriptional upregulation. HIF-1α will then induce various pathways which influence survival, proliferation, apoptosis, intratumoral angiogenesis, and many other processes.[6,17]

Statistical analysis showed that there were significant differences of in CXCR4 expression in laryngeal SCC with nodal metastasis compared to laryngeal SCC without nodal metastasis (p<0,05). Previous studies on laryngeal and head and neck SCC were also found similar results.[18-20] A study by Toyoma et al even proved that inactivation of CXCR4 will inhibit invasion and migration of tumor cells in SCC hypopharynx.[21]

Overexpression of CXCR4 is found in many malignancies. This is caused by CXCR4 upregulation by HIF-1α and HIF-2 secreted by malignant cells as a response to their increased oxygen demand.[7] CXCR4 upregulation can also be caused by DNA demethylation of CXCR4 promotor, a particular process detected in various types of cancer.[22] CXCR4-CXCL12 binding can increase MMP2 expression and inhibit tissue inhibitors of MMP. In addition, CXCL12 can promote the separation of tumor cells from tumor tissue and activated several cell adhesion molecules, and induce them to secrete more MMP and VEGF to dissolve ECM. These mechanisms contribute to tumor growth, angiogenesis, and metastasis.[23] Biasci et al, proved that inhibition of CXCR4 combined with blockade of PD-1/PD-L1 T cell check points can induce T lymphocyte infiltration, resulting in an anti-cancer response.[24] A study conducted by Tulotta et al showed that CXCR signaling supports the interaction between tumor cells and host neutrophils in developing tumor metastasis.[25]

This study also showed a significant difference in MMP13 expression in laryngeal SCC with nodal metastasis compared to laryngeal SCC without nodal metastasis (p<0,05). In this study, the median MMP13 expressions were stronger in laryngeal SCC with nodal metastasis group. Previous studies in laryngeal SCC.[26,27] The study conducted by Huang showed that siMMP13 knockdown not only reduced the tumor invasion and migration, but also decreased the adhesion abilities of oral cancer cells. It is supported that MMP13 promotes invasion and metastasis in oral cancer cells.[28]

MMP13 has a wide range proteolytic capacity. It is produced and secreted from many cell types as a precursor form (pro MMP13), which can be activated by plasmin, MMP2, MMP3, and MMP14. MMP13 or collagenase-3 has the ability to degrade not only fibrillary collagen (type I, II, III, V, and XI), but also basal membrane, cartilage, collagen type IV, IX, X and XIX, gelatin, fibrillin-1, tenascin, aggrecan, perlecan fibronectin, and osteonectin. MMP13 is expressed physiologically in conditions where rapid and effective remodelling of ECM are necessary, such as in fetal bone development and adult bone remodelling, and gingival and foetal skin wound healing. MMP13 is expressed pathologically in conditions involving overdegradation of ECM such as in rheumatoid arthitis, atherosclerosis, osteoarthritis and malignancy.[29-31]

In laryngeal SCC, tumor cells proliferate overwhelmingly. Tumor cells will secrete proteolytic enzymes and induce stromal cells, fibroblasts and inflammatory cells, in particular tumor associated macrophage (TAM), to produce MMP. MMP13 is a central key for other MMP cascade activation like MMP2, MMP3 and MMP9. All MMPs will then degrade ECM and basal membrane components together, facilitating tumor cells to invade stromal tissue and metastasize to lymph nodes and distant organs.[32,33]

Spearman correlation test showed no significant correlation between HIF-1α and CXCR4 expression, p = 0,403 (p>0,05). This finding is inconsistent with previous studies that showed a positive correlation between HIF-1α and CXCR4 expression in many types of malignancy, and also in laryngeal SCC.[14,34]

HIF-1α expression of laryngeal SCC tumor cells in this study tends to be low, while CXCR4 tends to be overexpressed. HIF-1α is known to be a major regulator

for CXCR4 upregulation, hence a low expression of HIF-1α should be followed by low expression of CXCR4. This contradictory finding could be caused by other molecular mechanisms which can also alter both protein levels, since molecules and biomarkers interactions in carcinogenesis are very complex and dynamic processes. As mentioned before, CXCR4 is not regulated solely by HIF-1α. Many malignancies had DNA demethylation of CXCR4 promotor, this will also upregulate CXCR4. Changes in multiple growth factors and transcription factors were also upregulate of CXCR4.[22,35] A recent study from Izumi et al also found that CXCR4 could be secreted by stromal myofibroblasts, known as cancer-associated fibroblasts (CAFs).[36]

In this study, Spearman correlation test showed positive correlation between HIF-1α and MMP13 expression in laryngeal SCC, r_s = 0,499 ($p < 0,05$). The stronger HIF-1α expression, the stronger MMP13 expression.

Hypoxia condition with high HIF-1α level will influence many mechanisms for tumor cells survival. HIF-1α regulates some proteins such as protein Twist, MMP-2, MMP-9, VEGF, and CXCR4/SDF-1. Matrix metalloproteinase like MMP-2, MMP-9 and MMP13 will degrade ECM surrounding tumor cells, eventually facilitate invasion of tumor cells through basal membrane into lymph and blood vessels to metastasize to lymph node and distant organs.[37]

In this study, Spearman correlation test also found positive correlation between CXCR4 and MMP13 expression, r_s = 0,409 ($p < 0,05$). The stronger CXCR4 expression, the stronger MMP13 expression. Previous studies also found similar results, not only in laryngeal SCC (Tan et al., 2008), but also in other malignancies such as basal cell carcinoma and colorectal cancer.[38,39]

CXCR4 activation will induce various downstream signaling pathways, induce EMT (epithelial to mesenchymal transition), and promote metastasis. Activated CXCR4 will dissociate to α and βγ subunit. Bγ subunit will activate phospholipase C-β (PLC-β) and PI3K. PLC-β will then divide phosphatidylinositol into IP3 (inositol (1,4,5) triphosphate and diacylglycerol (DAG). IP3 will induce intracellular calcium release and then together with DAG will activate protein kinase C and MAPK/ERK pathway.[40] MAPK/ERK then will relay the signal to induce MMP activity for ECM degradation by promoting transcription and translation of proMMP13 to MMP13.[22]

Regulation of MMP13 expression by CXCR4 has been proven by Bu et al. They analyzed silencing effect of CXCR4 gene in some signaling pathways, and found a significant decrease in ERK phosphorylation level, mRNA and MMP13 protein level. This proved that CXCR4 could regulate MMP13 transcription level through ERK/NFκβ pathway.[39]

Conclusion

There were significant differences among HIF-1α, CXCR4, and MMP13 expression in laryngeal SCC with nodal metastasis compared to laryngeal SCC without nodal metastasis. All three were expressed stronger in laryngeal SCC with nodal metastasis group. There was a positive correlation between HIF-1α and MMP13 expression, positive correlation between CXCR4 and MMP13 expression, but there was no correlation between HIF-1α and CXCR4 expression.

Conflict of Interest: The authors declare that they have no competing interest

Ethical Clearance: This study had been approved by RSUD Dr. Soetomo, Surabaya, Indonesia No. 474/Panke.KKE/VIII/2017

Source of Funding: Independent

Acknowledgements: The authors are thankful to The Anatomical Pathology Laboratory of Dr. Soetomo General Academic Hospital in Surabaya for providing the necessary facilities for this experiment.

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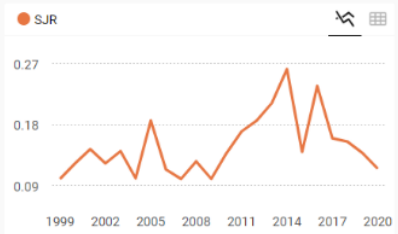

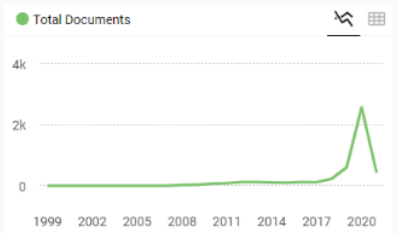

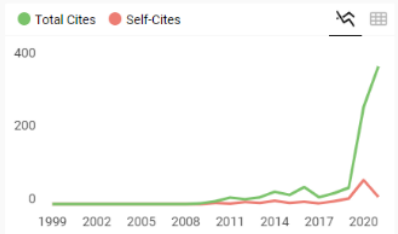

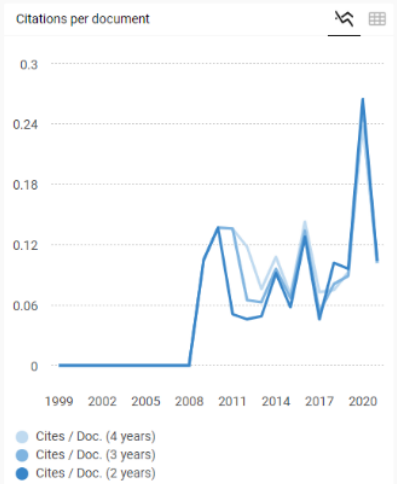

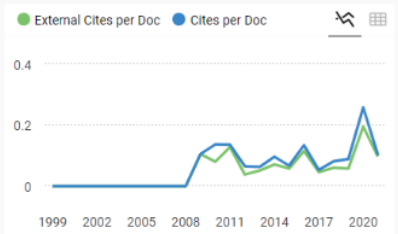

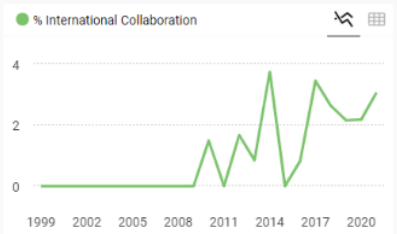

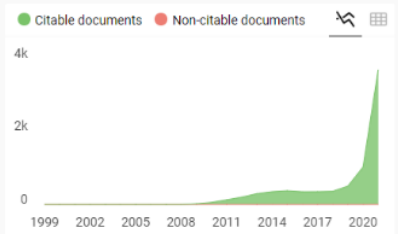

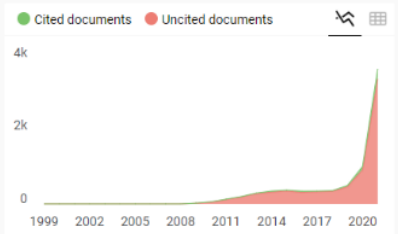

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