Salivary VEGF - A Potential Breast Cancer Marker

Subhajit Mondal[1], Sayantan Santra[1], Md Nasim Ali[2], Syamsunder Mandal[3], Ramanuj Mukherjee[4], Anjan Adhikari[4] & Amitava Chatterjee[1]

[1]Ramakrishna Mission Vivekananda University, India

[2]B.C.K.V., Chittaranjan National Cancer Institute, India

[3]Chittaranjan National Cancer Institute, India

[4]R G Kar Medical College, Kolkata, West Bengal, India

Correspondence: Dr Amitava Chatterjee, Ramakrishna Mission Vivekananda University, India. E-mail: amitavachatterjee24@gmail.com

Abstract

Aims: To identify and estimate salivary VEGF of breast cancer patients using non invasive method. Methods: ELISA, Zymography, Immunoblot. Results: We observed that saliva of breast cancer patients express appreciably high VEGF compare to non breast cancer saliva samples. Conclusion: The findings indicate salivary VEGF as a potential breast cancer marker using non invasive method.

Keywords: VEGF, MMP-2, MMP-9, Saliva, breast cancer

1. Introduction

Angiogenesis is essential for the growth of most primary tumors. Tumors can absorb sufficient nutrients and oxygen by simple diffusion up to a size of 1–2 mm, at which point their further growth requires the elaboration of a vascular supply. Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis (the growth of blood vessels from pre-existing vasculature) the effects of which on cancer growth and development have been well characterized (Folkman, 1995; Senger et al., 1983; Stuttfeld & Ballmer-Hofer, 2009; Folkman & Shing, 1992; Ferrara, 2002).

VEGF is a signaling homodimeric glycoprotein with molecular weight of 45 kD. VEGFs are predominantly produced by endothelial, hematopoietic, and stromal cells in response to hypoxia and upon stimulation by growth factors such as transforming growth factor beta (TGFβ), interleukins, or platelet-derived growth factors (PDGFs) VEGF A, which is often referred to as VEGF (in general), has been studied more than the other members of this family and it has several distinct isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$) because of alternative splicing of mRNA from a single, 8-exon *VEGFA* gene (chromosome 6p) (Ferrara & Alitalo, 1999; Carmeliet, 2002). During angiogenesis, it increases migration and mitosis of endothelial cells, matrix metalloproteinase activity, αvβ3 activity, creation of blood vessel lumen. VEGF family members elicit their effects on endothelial cells by binding to and activating tyrosine kinase receptors (Type-III) located on the cell surface. VEGF-A is capable of binding to multiple receptors, including VEGF receptor-1 (VEGFR-1/ Flt1) and VEGF receptor-2 (VEGFR-2/ Flk1) (Ortega et al., 1999; Ferrara et al., 2003). These are tyrosine kinase receptors that contain seven immunoglobulin-like domains on the extracellular portion of the receptor, as well as a single transmembrane region and an intracellular tyrosine kinase domain. These two receptors differ in their ligand binding properties and tyrosine kinase activity (Neufeld et al., 1999). VEGFR-1 binds to the VEGF ligand with higher affinity, whereas VEGFR-2 exhibits stronger inherent tyrosine kinase activity[ref]. Moreover, in patients with lung cancer, an increase in VEGFR2 gene copy number is associated with chemoresistance and shorter survival. Binding of VEGF to VEGFR-2 results in autophosphorylation of the tyrosine residues: Tyr951, Tyr1054, Tyr1175 and Tyr1214. Autophosphorylation of Tyr951 creates a binding site for the VEGFR-associated protein and Tyr1175 creates a binding site for Sck and PLCY1 (Matsumoto & Claesson-Welsh, 2001). VEGFR-2 also activates phosphatidylinositol 3-kinase (PI3K), which results in an increase of the lipid phosphatidylinositol (Takahashi et al., 1999) P3, leading to activation of protein kinase B (Akt/PKB), endothelial nitric oxide synthase, and the small GTP-binding protein Rac. When the cell becomes hypoxic, HIF1α persists and the HIF1α/β complex stimulates VEGF release (Chambers &

Matrisian, 1999). VEGF/VEGFR signaling also plays a vital role in cancer. Generally, VEGF produced by tumor cells is accepted to act on neighboring VEGFR-expressing ECs to promote neovascularization for continued tumor growth (Chambers & Matrisian, 1999). Under hypoxic conditions, induction of VEGF secretion was similarly inhibited by VEGFR2 inhibition, suggesting that this autocrine loop is also active in the physiological response to hypoxia (Chambers & Matrisian, 1999).

Matrix metalloproteinases, a large family of calcium dependent zinc containing endopeptidases, capable of degrading different kinds of extracellular matrix proteins (Abraham et al., 2007). In the process of angiogenesis, several MMPs are important but most importance is given to MMP-2 & MMP-9. They can degrade basement membrane components such as type-IV collagen, unmask cryptic biologically relevant sites in ECM components, modulate angiogenic factors, and are involved in the production of endogenous angiogenic inhibitors. The MMP-2 (72 kD) and MMP-9 (92 kD) have been shown to play critical roles in the "angiogenic switch" and tumor cells could synthesise and secret large amounts of MMP-2 and MMP-9 in a paracrine and/or autocrine manner to stimulate angiogenesis releasing VEGF (Christodoulides et al., 2007). A strong correlation of MMP-2 activity and increase of VEGF has been reported (Abraham et al., 2007).

Using salivary diagnostics, molecular diagnostics and nanotechnology Saliva is clinically very informative biological fluid that is useful for novel approaches to clinical diagnosis and prognosis monitoring and management of patients (Abraham et al., 2007; Christodoulides et al., 2007). Saliva can be collected using non invasive method, stored and ideal for early detection of disease because many of the biomarkers are present in saliva. Salivary diagnostics received wide attention (Gau & Wang, 2007). Salivary diagnostics are being developed in chronic obstructive pulmonary disease (COPD) and cystic fibrosis, acute myocardial infurction (Walt et al., 2007) and detection of oral cancer. Reports are there of elevated level of CA15-3 and the oncogene c-erB-2 in breast cancer patients[ref]. The biomarkers present in blood and urine can also be detected in a sample of saliva (Bhattacharyya et al., 2017). It has been reported by Pammer et al (Pammer et al., 1998) and Taichman et al (Taichman et al., 1998), the presence of VEGF in saliva of healthy individual. Brooks et al has reported elevation of salivary protein factors in breast cancer patients (Brooks, 2008).

In the present communication, we report that in biological fluid like saliva of breast cancer patients, VEGF expression is appreciably high compare to non breast cancer patients making VEGF a potential breast cancer marker using non invasive method.

2. Materials

MMP-2, MMP-9 and VEGF monoclonal antibodies were purchased from Santa Cruz, USA. Protease inhibitor cocktail, TMB and NBT/BCIP were purchased from Roche, Germany. All other chemicals like Gelatin, acrylamide, bis-acrylamide, SDS, TRIS *etc* were purchased from Sigma, USA.

2.1 Patients

Saliva (500 ul) was collected from 18 female breast cancer patients before surgery and Saliva from different cancer patients other than breast cancer were also collected from R G Kar Medical College, Kolkata following the ethical guidelines. The samples were collected in autoclaved tubes, centrifuged at 5000 rpm for 15 mins at 4 °C and clean supernatants were stored (adding protease inhibitors cocktail) at - 20 °C until use.

2.2 Statistical Analysis

Statistical Analysis was performed with help of Epi Info (TM) 3.5.3. EPI INFO is a trademark of the Centers for Disease Control and Prevention (CDC). Descriptive statistical analysis was performed to calculate the means with corresponding standard error (s.e.). Also One Way Analysis of variance (ANOVA) followed by *post hoc* Tukey's Test was performed with the help of Critical Difference (CD) or Least Significant Difference (LSD) at 5% and 1% level of significance to compare the mean values. $p < 0.05$ was taken to be statistically significant

3. Methods

3.1 ELISA

25 ug of salivary proteins was coated per well (in triplicate per sample). The volume was adjusted to 50 ul and the ELISA plate was incubated at 37°C for binding of the antigen, The blank surface of the well was then blocked with 3% BSA for 1 hr at 37°C. 100 ul primay antibody to VEGF (1:2000 dil) was then added per well and incubated for 1 and half hrs at 37°C followed by diluted (1: 2000) second antibody coupled to HRP for 1 hr at 37°C. The wells were washed and colour was developed by TMB. The colour was read at 450 nM (Bhattacharyya et al., 2017).

3.2 Substrate Zymography

200 ug salivary proteins were separated in a 0.1 % gelatin impregnated 8 % SDS-PAGE for 2 hrs at 15 mA. The gel was washed in 2.5 % Tween-20 for 1 hr. The gel was then incubated in buffer (NaCl 0.2M, CaCl2 4.5 mM, Tris 50mM, pH 7.4) keeping at 37°C for 15 hrs. The gel was then stained with Coommassiee Brilliant blue to detect the bands (Bhattacharyya et al., 2017).

3.3 Immunoblot

200 ug of salivary proteins were run in SDS- PAGE and the proteins were blotted on Nitrocellulose membrane. The NC paper was blocked with BSA and reacted with anti VEGF, MMP-9 or MMP-2 antibody followed by secodary antibody coupled to alkaline phosphatase. The colour was developed by NBT/BCIP (Bhattacharyya et al., 2017).

4. Results

4.1 Salivary VEGF, MMP-2 and MMP-9 in Breast and non Breast Cancer Samples

Figure 1 A clearly shows that salivary VEGF were much higher in breast cancer patients compare to non breast cancer patients. Figure 1B shows that the salivary MMP-2 is also high in saliva of breast cancer patients compare to non breast cancer patients. In case of MMP-9 the relation is just opposite. (Figure 1 C). shows MMP-9 concentration is less in breast cancer saliva samples compare to non breast cancer samples. The ELISA were done following the method described in method section.
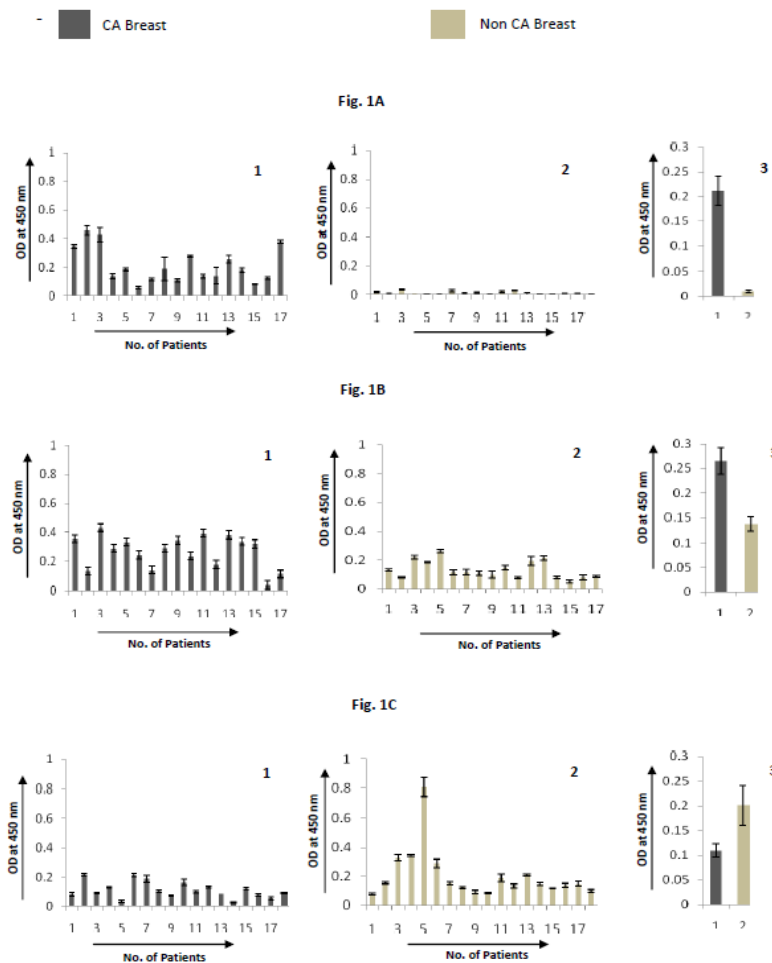

Figure 1. Assay of VEGF/ MMP-2 & MMP-9 by ELISA- [A] shows the salivary VEGF ELISA reading of breast cancer patients (1), Non breast cancer patients saliva (2) and average readings (3). Figure 1 [B] shows the salivary MMP-2 ELISA of breast cancer patients (1) non breast cancer patients (2) and average readings (3)., Figure 1 [C] is the MMP-9 ELISA reading of breast cancer patients (1), non breast cancer patients (2) and comparative average readings of breast and non breast cancer patients

4.2 Salivary MMP-2 Activity in Breast and non Breast Cancer Samples

The salivary MMP-2 activity of breast cancer patient and non breast cancer patient was developed by zymography. The comparative zymogram (Figure 2) clearly shows that the salivary MMP-2 of breast cancer patients are highly active compare to non breast cancer saliva.
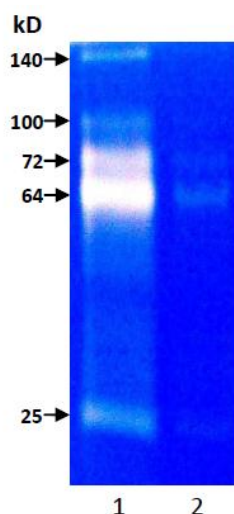

Figure 2. Comparative zymogram of saliva of breast (1) and non breast cancer patients (2). 200 ug of salivary proteins were subjected to develop zymogram following method described in method section

4.3 Immunoblot of Salivary VEGF, MMP-2 and MMP-9

Immunoblot of salivary proteins clearly shows that MMP-2 and MMP-9 are activated in breast cancer saliva (Fig 3). The immunoblot also shows the salivary VEGF at 45 kD region along with the processed product a 25 kD band.
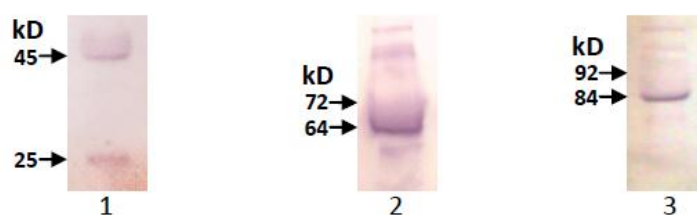

Figure 3. Immunoblot developed against salivary VEGF (1), MMP-2 (2) and MMP-9 (3). 200 ug of salivary proteins were run in SDS-PAGE, proteins were transferred to nitrocellulose membrane and reacted with anti VEGF, anti MMP-2 or anti MMP-9 antibody followed by ALP coupled 2nd antibody. The colour was developed with NBT/BCIP following standard method

Table 1. Comparative ELISA values of salivary VEGF of patients with breast cancer, other than breast cancer and non-cancer patients

Group	OD (Mean±s.d.)
Breast Cancer	0.2118±0.1237
Other than Breast Cancer	0.0346±0.0266
Non cancer	0.0060±0.0153

One way ANOVA showed that there was significant difference in OD values of the subjects of the three groups ($F_{2,50} = 39.94$; $p<0.001$). As per CD the mean OD of the patients with breast cancer was significantly higher than the other groups. Also the mean OD of the patients with other than breast cancer was significantly higher than that of non-cancer patients.

5. Discussion

Vascular Endothelial Growth Factor (VEGF) is one of the main regulators in angiogenesis and neovascularization. It has been reported that plasma or serum VEGF may be used as breast cancer marker (Iyer et al., 1998). We observed that VEGF is much higher in amount in saliva of breast cancer patients compare to non breast cancer patients. So far we measured salivary VEGF in 18 breast cancer patients. It has been reported that MMPs has very close association with the VEGF activity (Byrne et al., 2007). We observed MMP-2 and 9 both in expression and activity level. Interestingly we observed that MMP-2 is expressed in much higher level in saliva of breast cancer patients compare to non breast cancer patients. In expression level MMP-9 shows less in amount in breast cancer patients saliva compare to non breast cancer patients. In immunoblot MMP-9 is also focused at activated (84 kD) region. This relation of activated MMP-2 / MMP-9 are very meaningful because the role of MMPs are important during invasion of endothelial cells for neovascularization (Brooks et al., 2008; Chetty et al., 2010; Mazure et al., 1997).

The metastatic progression is a complex multi-step process involving interactions between cancer cells and their microenvironment. During the growth of primary tumor above 1 cmm the angiogenic blood vessels stimulate to grow to feed the cancer cells for tumor growth and spread to secondary sites. For growth of this vasculature the role of MMP (2/9) are very important. The immublot of salivary proteins of breast cancer patients show that the 45kD VEGF has been processed to 22 kD band (Figure 3). So increase of VEGF and MMP-2 expression & activity in saliva of breast cancer patients perhaps indicate the interdependence of MMP-2 and VEGF (Walt et al., 2007; Bhattacharyya et al., 2017).

Saliva is a biological fluid having potential to find out important biomolecules which may be used as biomarkers. Here we found that VEGF which is one of the important regulators of angiogenesis could be used as possible breast cancer marker using non invasive method.

Conflict of Interest

The authors confirm that this article content has no conflict of interest.

Acknowledgements

References

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med., 1*, 27-31. https://doi.org/10.1038/nm0195-27

Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., & Dvorak, H. F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science, 219*(4587), 983-5. https://doi.org/10.1126/science.6823562

Stuttfeld, E., & Ballmer-Hofer, K. (2009). Structure and function of VEGF receptors. *IUBMB Life, 61*(9), 915-922. https://doi.org/10.1002/iub.234

Folkman, J., & Shing, Y. (1992). Angiogenesis. *J Biol Chem., 267*, 10931-10934.

Ferrara, N. (2002). VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer, 2*, 795-803. https://doi.org/10.1038/nrc909

Ferrara, N., & Alitalo, K. (1999). Clinical applications of angiogenic growth factors and their inhibitors. *Nat Med., 5*, 1359-1364. https://doi.org/10.1038/70928

Carmeliet, P. (2002). Mechanisms of angiogenesis and arteriogenesis. *Nat Med., 6*, 389-395. https://doi.org/10.1038/74651

Ortega, N., Hutchings, H., & Plouet, J. (1999). Signal relays in the VEGF system. *Front Biosci., 4*, D141-D152.

Ferrara, N., Gerber, H. P., & LeCouter, J. (2003) The biology of VEGF and its receptors. *Nat Med., 9*, 669. https://doi.org/10.1038/nm0603-669

Neufeld, G., Cohen, T., Gengrinovitch, S., & Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J., 13*, 9-22. https://doi.org/10.1096/fasebj.13.1.9

Matsumoto, T., & Claesson-Welsh, L. (2001). VEGF receptor signal transduction. *Science STKE, 112*(RE21), 1-17. https://doi.org/10.1126/stke.2001.112.re21

Takahashi, T., Ueno, H., & Shibuya, M. (1999). VEGF activates protein kinase C dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene, 18*, 2221-2230. https://doi.org/10.1038/sj.onc.1202527

Chambers, A. F., & Matrisian, L. M. (1999). Changing view of the rol of MMPs in metastasis, *J Natl Cancer Inst., 89*, 1260-1270. https://doi.org/10.1093/jnci/89.17.1260

Abraham, W. R., Barber, C. A., McCann, K. et al (2007), Development of a microfluidic device for detection of pathogens in oral samples using upconverting phosphor technology, *Ann N Y Acad Sci., 1098,* 375-388. https://doi.org/10.1196/annals.1384.020

Christodoulides, N., Floriano, P. N., Miller, C. S. et al. (2007). Lab on a chip methods for point of care measurements of salivary biomarkers of periodontitis, *Ann of N Y Acad Sci., 1098*, 411-428. https://doi.org/10.1196/annals.1384.035

Gau, V., & Wang, D. (2007). Oral fluid nanosensor test with advanced electrochemical based molecular analysis platform, *Ann N Y Acad Sci., 1098*, 401-410. https://doi.org/10.1196/annals.1384.005

Walt, D. R., Blicharz, T. M., Heyman, R. B. et al. (2007). Microsensor Arrays for saliva diagnostics, *Ann N Y Acad Sci., 1098,* 389-400. https://doi.org/10.1196/annals.1384.031

Bhattacharyya, N., Mondal, S., Ali, N. M., Mukherjee, R., Adhikari, A., & Chatterjee, A. (2017). Activated Salivary MMP-2 - A Potential Breast Cancer Marker. *The Open Conference Proceedings Journal, 8*, 3-13. https://doi.org/10.2174/2210289201708010022

Pammer, J., Weininger, W., Mildner, M., Burian, M., Wotja, J., & Tschachler, E. (1998). Vascular Endothelial Growth Factor is constitutively expressed in normal human salivary glands and is secreted in the normal saliva. *Jour of Pathology, 186*, 186-191. https://doi.org/10.1002/(SICI)1096-9896(1998100)186:2<186::AID-PATH148>3.0.CO;2-J

Taichman, N. S., Cruchley, A. T., & Flecher, L. M. (1998). Vascular Endothelial Growth Factor in normal human salivary glands and saliva. A possible role in the maintenance of homeostssis, *Lab Invest, 78*, 869-875.

Brooks, M. N., Wang, J., Li, Y., Zhang, R., Elashoff, D., & Wang, D. T. (2008). *Molecular Med, I*, 375-378.

Chetty, C., Lakka, S., Bhoopathi, P., & Rao, J. (2010). MMP-2 Alters VEGF Expression via αVβ3 Integrin-Mediated PI3K/AKT Signaling in A549 Lung Cancer Cells. *Int J Cancer, 127*(5), 1081-1095. https://doi.org/10.1002/ijc.25134

Mazure, N. M., Chen, E. Y., Laderoute, K. R., & Giaccia, A. J. (1997). Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood, 90*, 3322-3331.

Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V., & Ferrara, N. (1998). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem., 273*, 30336-30343. https://doi.org/10.1074/jbc.273.46.30336

Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., … Semenza, G. L. (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev., 12*, 149-162. https://doi.org/10.1101/gad.12.2.149

Byrne, G. J., McDowell, G., Agarawal, R., Sinha, G., Kumar, S., & Bundred, N. J. (2007). Serum Vascular Endothelial Growth Factor in Breast Cancer. *Anti Can Resh, 27*(5B), 3481-7.

Consetta, S., Andrea, M., Girolamo, R., Katia, D., Giovanni, S., Angelo, P., & Anita, M. (2013). VEGF, HIF-1alfaExpression and MVD as an Angigenic network in Familial Breast Cancer, Plos One, January.

Adams, J., Carder, P. J., Downey, S., Forbes, M. A., MacLennan, K., Allagar, V., & Banks, R. E. (2000). Vascular Endothelial Growth Factor (VEGF) in Breast Cancer: Comparison of plasma, serum and tissue vegf and microvessel density and effects of tamoxifen. *Can Resh, 6*(11), 2896-905.

Copyrights