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A facile gold nanoparticle–based ELISA system for detection of osteopontin in saliva: Towards oral cancer diagnostics

Debolina Chakraborty[a], Thangaraj Soundara Viveka[b], Krishnamurthy Arvind[b], Vidyarani Shyamsundar[c], Murhekar Kanchan[b], Sruthi Ann Alex[a], N. Chandrasekaran[a], Ramshankar Vijayalakshmi[b,*], Amitava Mukherjee[a,*]

[a] Centre for Nanobiotechnology, VIT University, Vellore, India
[b] Department of Preventive Oncology (Research and Molecular Diagnostics), Cancer Institute (WIA), Chennai, India
[c] Centre for Oral Cancer Prevention Awareness and Research, Sree Balaji Dental College and hospital, Bharath University, Chennai, India

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ABSTRACT

In the current study, we emphasize that osteopontin is overexpressed in oral squamous cell carcinoma. Overexpression of osteopontin levels was confirmed by mRNA quantification studies and immunohistochemistry analysis. Based on this, a gold nanoparticle–based ELISA system was developed for non-invasive osteopontin detection. The incorporation of AuNRs (Gold nanorods) or AuNSs (Gold nanospheres) in the conventional ELISA improved the sensitivity of analyses. A considerably lowered detection limit in case of AuNR (detection limit: 0.02 ng mL^{-1}) and AuNS (detection limit: 0.03 ng mL^{-1}) modified assay was obtained as compared to commercially available OPN ELISA kit (detection limit: 0.14 ng mL^{-1}). The modified ELISA had a wide linear detection range (0.31–20 ng mL^{-1}), good reproducibility, and specificity against the tested interferents in the saliva. Finally, the nanoELISA was validated with osteopontin spiked in artificial and normal saliva samples and observed to show good recovery (95.4–97.85%), which indicates the application potential of the developed kit for real sample analysis.

1. Introduction

Oral cavity cancer is the eight most common cancers worldwide with a high prevalence among men. There are approximately 300,000 new cases annually worldwide [1,2]. In south central Asia, oral cancer ranks among the three most common types of cancer. The age standardized incidence rate of oral cancer is 12.6/100,000 in India. Oral cancer survival rate is < 60% and there has been no improvement in survival for the past 5 decades [3], due to late diagnosis frequently in up to 50% of the patients with lymph node metastasis during presentation [4]. Initial staging of oral cancer based on TNM (T stands for "Tumour Size" – size of the primary tumour, measured in cm or mm; N stands for "Nodes" – indicating the extent of spread of the cancer to the regional lymph nodes and M stands for "metastasis" – indicating if the cancer has spread to the other organs of the body from its primary location) classification, which is currently in practice, appears insufficient to accurately predict the prognosis and is not adequately helpful to tailor treatment. Biomarkers are therefore an important need to predict treatment response and to customise therapy options [5]. Since most of the Oral Squamous cell carcinoma (OSCC) develops from visible lesions in the oral cavity, the most preferred biomarker detection medium includes biological fluids like blood and saliva. Saliva holds a promising future in search of newer clinical markers as it is easily accessible, non-invasive, accurate and cost effective to investigate the malignant molecular pathology by secreted form of biomarkers from tumour.

Osteopontin (OPN) is an extracellular matrix (ECM) associated cytokine like sialic acid rich phosphoglycoprotein [6–8]. It is member of the SIBLING (small integrin binding ligand and N-linked glycoprotein) family. Several recent reports reveal that osteopontin is expressed in tumour educated stromal cells and leads to cancer progression [9]. It plays an important role in tumour invasion, tumour growth, angiogenesis and metastasis by up regulating several signalling pathways that lead to overexpression of target proteins like Matrix Mettaloproteins 2/9, urokinase plasminogen activator (uPA) and vascular endothelial growth factor (VEGF) [10]. However, OPN is important for normal physiological processes like wound healing, bone resorption, tissue remodeling, immune responses, and vascularisation as well.

Recent clinical studies show that OPN is overexpressed in tumour tissues and serum samples from patients of various cancers [11]. Elevated levels of OPN in plasma has been associated with unfavourable

* Corresponding authors.
E-mail addresses: r.vijayalakshmi@cancerinstitutewia.org (R. Vijayalakshmi), amitav@vit.ac.in (A. Mukherjee).

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outcome in several cancers [12]. OPN has been shown to have a definite prognostic significance in head and neck cancers patients treated with radiotherapy previously [13]. OPN is associated with tumour hypoxia and a malignant phenotype. Despite a large body of evidence to show OPN detection, there is still no validated and certified OPN ELISA based test that can be used for cross study comparisons. OPN values have been different significantly using different ELISA systems applied [14].

Nanoparticle-based sensing system has been used very frequently for cancer diagnosis [15] for detecting circulating tumour cells (CTC), circulating nucleic acids (CNA), circulating proteins [16], etc. Immunosensors made of L-cysteine-capped lanthanum hydroxide [17], nanostructutred zirconium oxide [18], and nanostructured hafnium oxide [19] have shown promising results in the detection of cyfra-21-1, an oral cancer biomarker. On the other hand, unique optical properties, improved biocompatibility, and modifiable surface chemistry of gold nanoparticles (AuNPs) have contributed remarkably in the development of nano-biosensors [20–23]. These gold NP-based biosensors utilize diverse principles such as SERS [24], interaction-based alteration in dynamic light scattering [25], colorimetric response [26], and many more for detecting cancer. Though these methods tend to enhance the sensitivity of the assay, a sensor that is more facile in nature as well as minimizes the instrumentation cost would be most preferable. Thus, conventional ELISA still remains one of the best analytical assays with high-end accuracy and least complexity involvement. The incorporation of NPs in conventional ELISA protocol can be useful in enhancing the sensitivity and shortening the incubation time of the assay [27].

In the current study, we developed a non-invasive AuNP-based ELISA for OPN detection in saliva samples with amplified response when compared with conventional ELISA without increasing the complexity of the pre-defined protocol. To the best of our knowledge, this is the first work that establishes OPN as a biomarker in saliva and summarises the comparative sensing capabilities of two different types of AuNP bioconjugates i.e. with gold nanospheres (AuNSs) and gold nanorods (AuNRs), for the development of nanoELISA for OPN detection. The work demonstrates that the use of AuNPs can considerably improve the limit of detection (LOD) when compared to the commercially available kit, paving the way for their probable application for oral cancer prognosis in future.

2. Materials and methods

2.1. Materials

Cetyltrimethylammonium bromide (CTAB), sodium borohydride ($NaBH_4$), N-hydroxysuccinimide (NHS), N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), sodium phosphate monobasic (NaH_2PO_4), tween-20, and 11- mercaptoundecanoic acid (MUA) were procured from Sigma-Aldrich (India). Trisodium citrate, hydrogen tetrachloroaurate hydrate ($HAuCl_4{\cdot}2H_2O$), and potassium chloride were purchased from SRL Pvt. Ltd. (India). Silver nitrate was purchased from merck. Sulphuric acid and ascorbic acid were procured from SD Fine Chemicals Ltd. (India). Sodium bicarbonate ($NaHCO_3$), potassium thiocyanate (KSCN), sodium chloride (NaCl), glucose ($C_6H_{12}O_6$), and blocking buffer were purchased from Himedia Laboratories Pvt. Ltd. (India). Human Osteopontin (OPN) Quantikine ELISA DOST00 kit was bought from R & D Systems, and osteopontin antibody (with HRP) was from Biorbyt Ltd. Glycine was procured from Himedia Laboratories Pvt. Ltd. (India) and α-amylase from human saliva type IX-A was purchased from Sigma-Aldrich (India). All the glassware was thoroughly cleaned with aqua regia ($HCl:HNO_3$ = 3:1), followed by rinsing and was finally dried in a hot-air oven.

Trizol (Invitrogen, Life Technologies, CA, USA) and RNeasy® Plus Mini kit (Qiagen, Hilden, Germany) was used for RNA extraction from tongue tissue samples. cDNA conversion using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. FastStart Universal SYBR Green Master (Rox) (Roche) was used according to the manufacturer's instructions on a 7500 Real Time PCR System (Applied Biosystems) for qPCR based quantitations.

2.1.1. Patient materials

Formalin-fixed paraffin sections (n = 146) were obtained from oral tongue cancer patients admitted to the tertiary care centre between 1995 and 2000 with their clinical information. Surgical margin tissues (n = 6) from prospective patients who underwent wide excision glossectomy and histological normal tissues (n = 2) were obtained to compare the OPN protein expression. Additionally, RNA of tumour tissues from prospective tongue cancer patients (n = 68) were collected after taking an informed consent from the patients as per the guidelines of Institutional Ethical clearance. Surgical margins were obtained from these patients (n = 17) and verified that they were histologically non-malignant. All research involving human participants had been approved by the author's Institutional Review Board (IRB), and all the clinical investigations had been conducted according to the principles expressed in the declaration of Helsinki. A written informed consent was obtained from all the participants, and the contents of the informed consent was approved by the IRB (Cancer Institute WIA; Protocol 1 HNCOG (Cancer Institute, Women's India Association; Protocol 1 Head and Neck Co-operative Oncology Network). The finger prints were obtained for patients, who were illiterate after explaining the protocol, and a written informed consent was additionally taken from patient's relative presenting as witness.

Unstimulated saliva was collected from a healthy volunteer after obtaining an informed consent from Institutional Ethical Committee Clearance.

2.2. OPN as a biomarker: methodology

2.2.1. mRNA extraction

Briefly, tissues were ground with a mortar and pestle with liquid nitrogen, and TRIzol was added to powdered tissue and mixed well. 200 μL of chloroform was added, homogenized, and centrifuged. The aqueous phase was transferred to a new tube, avoiding contact with the interface, and 500 μL of 100% isopropyl alcohol was added to it, and this was further processed using RNeasy® Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA samples were then stored at − 80 °C.

2.2.2. Real-time PCR analysis

Real-time PCR was performed to measure the mRNA expression of OPN in primary oral tongue squamous cell carcinoma tissues (n = 68), adjacent uninvolved non-cancerous tissue called apparently normals (n = 6), and absolutely normals (n = 11) as described. The primer sequences used for the study are shown in Table S1. The quantitative real-time RT-PCR was performed using FastStart Universal SYBR Green Master (Rox) (Roche) according to the manufacturer's instructions on a 7500 Real-Time PCR system (Applied Biosystems). Universal thermal cycling conditions used were as follows: 10 min at 95 °C, 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Data was collected at every temperature phase during each cycle. The comparative threshold cycle (Ct) method was used to calculate the fold change. β-Actin gene was used as a reference control to normalize the expression values.

Triplicates were performed for each gene, and the average expression value was computed for the subsequent analysis. The relative expression level of the genes was calculated using the ($2^{-\,ddct}$) method.

2.2.3. Immunohistochemistry (IHC)

The IHC detection of OPN expression was performed on five-micron sections of FFPE tissues. The sections were deparaffinised in xylene and rehydrated in absolute ethanol. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in distilled water for