Original Article

Effect of Herbal Anticoagulant on ctDNA Yield in Blood from Oral Squamous Cell Carcinoma Patients

Abstract

Oral squamous cell carcinoma (OSCC) is one of the most common cancers. It accounts for 90–96% incidence of all head and neck cancers. Understanding molecular pathogenesis can pave the way for precision-based treatment as well as improve the quality of life. The aim of the present study was to investigate the effects of Gymnema sylvestre (GS) and Ethylenediaminetetraacetic acid (EDTA) on ctDNA yield in OSCC. The prospective study was conducted on 20 primary OSCC patients. The blood samples for patients taken and was preserved in tubes containing GS and EDTA. Polymerase chain reaction (PCR) was carried out to quantify the ctDNA. EDTA and GS have almost similar effects and similar yields on ctDNA from the blood of OSCC. It was concluded that apart from chemical anticoagulants like EDTA, GS can also be used as in-vitro laboratory anticoagulants for storing blood from OSCC, which can be used for molecular analysis.

Keywords: *EDTA, Gymnema Sylvestre, Anticoagulant, ctDNA, OSCC, Oral Squamous cell carcinoma*

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common head and neck cancers, accounting for 90–96%.[1] OSCC has poor life expectancy even after the best possible management of tumors. OSCC is frequently treated with surgery, chemotherapy with/without radiation. However, the average life expectancy in OSCC over 5 years stays from 40 % to 53.7% in the last few decades.[2] Further, it gets worse with the presence of metastasis, recurrence, and cancer stage. In addition, etiopathogenesis plays a vital role in the progression and outcome of OSCC.

Early detection is an effective method of reducing mortality and morbidity in OSCC.[3] It includes several clinical and radiological methods. However, it needs to be confirmed with histopathology examination, which is the least preferred method due to its invasive nature. Further, the patient may not be ready for a biopsy even if clinically it looks suspicious. By the time the patient, seeks medical treatment OSCC is already reached in late stage leaving behind only a salvaging option. Further, treatment response varies among the OSCC patient. One important reason is that the mechanism of squamous cell carcinoma is unclear.

Several tools are currently employed to comprehend the complexity of molecular pathogenesis.[4] Carcinogenesis involves the mutation and aberration in cells' genetic composition including cDNA.[5] Further, Understanding genetic mutation can improve and develop specific cancer therapies.[6] Currently, Oral cancers are treated with standard treatment options without personalized curative therapy. This is one reason for unpredictable outcomes in oral cancer patients. As a result, there is increased interest in the development of personalized gene-based treatment for a better outcome.

Researchers have been using several biological samples for studying molecular analysis. It included tissue, body fluid, etc. Tissue has the disadvantage of being an invasive procedure and can be less sensitive to early diagnosis. With the advent of sophisticated technology, non-invasive technique are evolved to improve precision. Several liquid-based biopsy methods have been developed to identify the mutation in oral cancer which mostly involve the study of ctDNA.[7] However, these have their challenges.[8] It involves sample processing, storage, transport, etc. that has been reported to affect the ctDNA yield.

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EDTA is the most common anticoagulant used for the collection of blood from cancer patients and was sent for ctDNA estimation. However, EDTA can affect the yield due to fragmentation for ctDNA. This poses difficulty in finding the mutation and aberration for further analysis. Currently used anti-coagulants are synthetic and often interfere with genetic components.[9] Lately, new anti-coagulant from natural sources such as plants are being explored. Considering the nature, these can have less effect on genetic structure.

Gymnema Sylvestre (GS) is a natural herbal plant with several pharmacological properties Further, it has been shown to affect the blood parameter in vivo.[10] It has good anticoagulant activity.[11] Hence; it was suggested as an alternative to EDTA. However, there is no information on whether it will interfere with DNA yield. In this study, we evaluated the DNA yield in blood samples collected and stored in GS extract compared to EDTA.

Materials and Methods

The prospective study was carried out on an OSCC patient who visited for Oncology treatment at the Hospital. Informed consent was taken from patients for using the blood for the investigation. The institute's ethics committee approved the study [IHEC/SDC/GPATH-2000/22/101].

Sample collection

A 2 ml sample of peripheral blood was collected from OSCC patients using standard precautions. Blood samples were separated equally and placed in tubes with GS extract or EDTA. After that, both samples with centrifuged at 12500 rpm for 15 minutes, and the plasma was separated. The plasma was stored at – 80°C. The DNA was extracted from plasma using a commercial DNA extraction kit. 100 µL purified DNA was diluted with 70 µL of buffer AE and stored at –20°C for further experiment.

cDNA quantification

The 100-µl sample was added to 1 ml Trizol reagent and homogenized. After that, 0.2 ml chloroform was added and mixed by vortexing for a few seconds. The samples were centrifuged at 12500 rpm for 15 mins and the supernatant was carefully pipetted. The supernatant was diluted with an equal volume of isopropyl alcohol and centrifuged at 8500 - 9000 rpm for 10 mins. 1 ml of 70% ethanol to the pellet was added and centrifuged at 7500 rpm for 5 mins. After that, the supernatant was removed and air-dried. The obtained RNA was dissolved with 100µl of sterile water (DEPC) and nanodrop quantification was performed.

Results and Discussion

Anticoagulants did not show a significant difference in cDNA yield **(Figure 1)**. GS had a mean concentration yield of 1022 ng/µL (SD=35.59 ng/µL), which was nearly the same as EDTA's mean concentration yield of 936.33 ng/µL (SD=128.47 ng/µL). Both EDTA and GS powder had similar effects on RNA concentration, RNA purity **(Figure 2)**, and cDNA in the blood of oral squamous cell carcinoma patients.

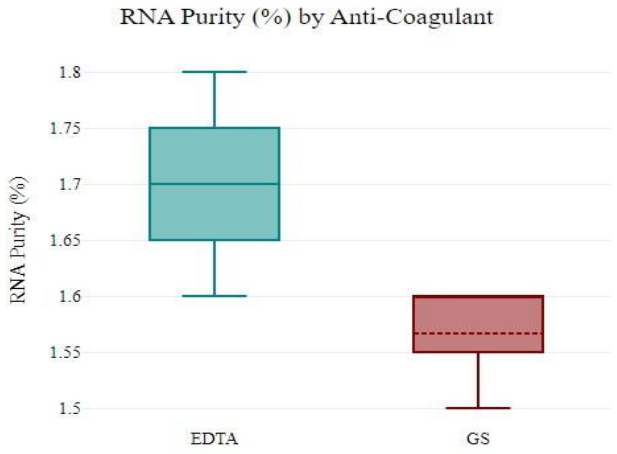

Figure 1. Comparison of GS and EDTA for RNA purity in the blood collected from OSCC patients.

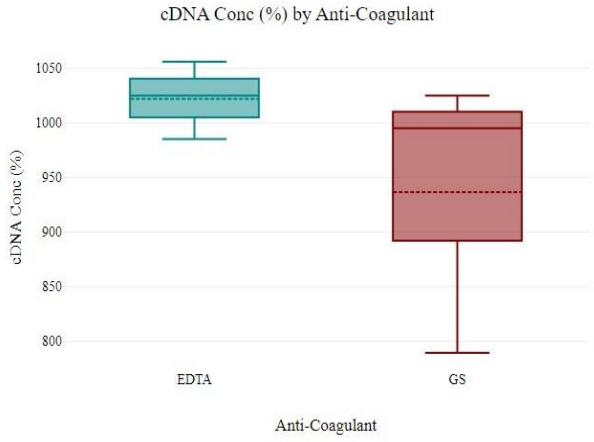

Figure 2. Comparison of GS and EDTA for ctDNA concentration in the blood collected from OSCC patients.

GS is versatile having several properties. Anti-coagulant is one such property that can be beneficial in the field of research. GS anticoagulant activity can be used for storing the blood sample that is used for molecular study considering its activity is similar to EDTA. In the present study, we observed that GS has little effect on cDNA yield and was equivalent to EDTA. However, we have used crude GS extract as an anti-coagulant, so there is more likelihood that further purified components may show better efficacy in terms of cDNA yield.

Previously, Gymnema species were suggested as an in-vivo anticoagulant for certain diseases owing to its natural source.[10] Although few studies are exploring the anti-coagulant activity, it was observed that it prolongs the PTT and APTT. Based on the concentration-dependent manner along with antimicrobial activity, GS shows significant anticoagulant activity. Leaf showed maximum anticoagulant activity of (23: 12 mins) in 500 ppm that shows very good anticoagulant activity that is almost similar to EDTA which is the standard anticoagulant drug.

The Anticoagulant activity of the compound usually depends on the chelation of inhibition of the enzyme involved in coagulation pathways.[12] There is clear information on by which mechanism the anti-coagulant activity of GS work. Certain plants such as *Jatropha gossypiifolia L. (Euphorbiaceae)* produce anticoagulant activity by enzymatic inhibition activity. Further, analysis of the crude extract of these plants with potential anticoagulant activity was found to contain protease inhibitors in CE, especially serine proteases inhibitors.[13] Similarly, the aqueous leaf extract of *Melastoma malabathricum Linn.* possesses potent anticoagulant properties.[14] Khoo *et al*. found that active anticoagulant fractions F1, F2, and F3, fractions F1 and F3 are present in *Melastoma malabathricum Linn* which are responsible for the anti-coagulant activity. Further, the analysis suggested the role of Subfraction B in anticoagulant activity.[14] Whereas another study on the anticoagulant activity of *artemisia dracunculus* leaf extracts reported the involvement of coumarin derivatives in the anti-coagulation activity of the extract.[15] However, in the case of GS, no compound was identified to be involved in anti-coagulant activity even though it contains several compounds having pharmacological action. Future studies should focus on identifying the compound in GS responsible for anticoagulant activity.

Conclusion

GS can prevent blood clotting without affecting the amount of ctDNA, which can enhance the quality of research. Future studies can explore how to modify GS to make it a natural alternative to synthetic anticoagulants.

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Conflict of interest

None

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Ethics statement

The study was approved by the Institutional Ethics Committee (IEC) (Number: IHEC/SDC/GPATH-2000/22/101).

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