

A STUDY OF RNASE ENZYMES IN SALIVA SAMPLES FROM WOMEN WITH BREAST TUMORS

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SUMMARY: RNase activities in saliva of 30 patients with breast benign tumors, 33 patients with breast malignant tumors and 25 healthy individuals were measured.

This study was devoted to the measurement of RNase activity (alkaline and acid) in saliva of control, benign and malignant groups. The results indicated the presence of a highly significant elevation ($p < 0.001$) of these enzyme's activities in cancer groups in comparison with those of other groups. Upon conventional electrophoresis of the crude saliva samples of the above three studied groups, differences in the patterns of proteins and RNase activity were recognized.

Key Words: Ribonuclease, Saliva, Breast cancer.

INTRODUCTION

Breast cancer is the most common cause of cancer death among women worldwide. The population of breast cancer increased more than double from the years 1991 to 2000 (1-4).

The vast bulk of biochemical components in tumor tissue are "normal," in the sense that they are produced by certain specialized adult normal cells or by normal cells at some stages of their differentiation. In cancer cells, it is the combination and proportions of these normal components that are abnormal. The biochemical diversity of cancer cells, then, would depend on the cell of origin of the neoplasm and its degree of neoplasticity (5).

Saliva as a diagnostic medium has many advantages over serum for a large variety of types of testing. Because saliva can be collected without breaking the skin or entering the body in any other way, it has obvious advantages for multiple noninvasive collections and for obtaining samples from those whom, for cultural reasons, or age, or because of physical or mental handicaps, it would be unethical to collect blood samples (6).

Saliva is a complex fluid produced by a number of specialized glands which discharge into the oral cavity of the glands of mammalian vertebrates. Most of the saliva is produced by the major salivary glands (parotid, submandibular, and sublingual), but a small contribution is made by the numerous small labial, buccal, and palatal glands which line the mouth (7-8).

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Table 1: Protein concentration, RNase activity and Specific activity in the saliva samples for the three studied group.

Groups	Samples	Age Years	Total protein mg/ml (mean \pm Standard deviation)	Activities U/L (mean \pm Standard Deviation)		Specific Activities U/mg (mean \pm Standard Deviation)	
				Alkaline RNase	Acid RNase	Alkaline RNase	Acid RNase
Control	25	23-71	(2.545 \pm 0.69)	(17.20 \pm 10.5)	(17.68 \pm 8.28)	(6.58 \pm 2.97)	(7.02 \pm 2.78)
Benign	30	21-69	(4.528 \pm 2.64)	(161.76 \pm 7.83)	(153.57 \pm 104.42)	(36.67 \pm 14.04)	(33.81 \pm 10.51)
Malignant	33	28-71	(9.72 \pm 2.06)	(842.32 \pm 40.47)	(837.325 \pm 274.46)	(87.11 \pm 19.26)	(85.19 \pm 16.81)

Mixed saliva containing (97-9.5)% water and the rest is solid. Solids are the organic substances and inorganic substances. Gases are also found in saliva (9).

Previously it was noted that saliva supplies enzymes for digestion. These enzymes and other proteins, including saliva-specific glycoproteins, are synthesized by the acinar cells. The transport of proteins into saliva has been reviewed by Young (10). Almost all of the organic compounds of plasma, such as hormones, immunoglobulines, enzymes, DNA and viruses may be detected in saliva in trace amounts (8).

Human ribonuclease is widely distributed in various organs (11) such as pancreas and body fluids (12), including serum, urine, saliva and cerebrospinal fluid.

According to the current situation of RNase, the relevant studies on this enzyme in the saliva indicated the importance of this enzyme in this biological fluid, as a diagnostic tool but most of them gave a special reference to the activity measurement and lack of some of the important biochemical and biological aspects.

As such the intention to study the following lines was arised:

1) Evaluating acid and alkaline RNase activities in saliva of the control women in comparison with that of the patients with benign and malignant breast tumors.

2) Detecting the changes in the forms of alkaline RNase enzyme that occurred upon malignancy.

MATERIALS AND METHODS

This study included two groups of breast tumor patients, 30 cases benign and 33 cases malignant serous breast tumor in different stages. These two groups were matched with a group of age matched healthy individuals (25 control), who were used as a control group.

All patients were admitted to; AL-Ilwia Hospital, Center of Early Detection of Breast Cancer. They were histologically proven under the supervision of specialists: - Dr. Perlant Asker Mahmood applying Fine Needle Aspirate (FNA) cytological study for diagnosis of breast cancer.

Saliva samples were collected in the morning from the patients and healthy women after thoroughly rinsing the mouth without any stimulation. The saliva was centrifuged (2000*g) for 10 min after collection and the supernatant was stored at (-20) $^{\circ}$ C until being used for different investigations. A modified Lowry method by Hartree (13) was used to determine saliva total proteins using Bovine Serum Albumin (BSA) as a standard.

Determination of alkaline RNase activity in the saliva samples

Determination of alkaline RNase activity in saliva was carried out according to Bardon et.al method (14) with some modifications. Reaction mixture 1.1 ml containing 1 ml buffered substrate solution (Davis buffer)(15) and a volume of saliva which contain 100 μ g of total protein were incubated for 15 min at 37 $^{\circ}$ C. After the incubation period, the reaction mixture was cooled to 0 $^{\circ}$ C and mixed with an equal volume of HCl (1 M) in 70% ethanol and left for 30 min. The samples were centrifuged at (2000*g) for 10 min. and the supernatant was diluted with distilled water (1:5). The absorbencies were measured at (λ =260)

Figure 1: Conventional-PAGE electrophoresis profile of crude saliva, electrophoresis was carried out using 12.5% acrylamide, 30mA current, the gel was stained for protein.

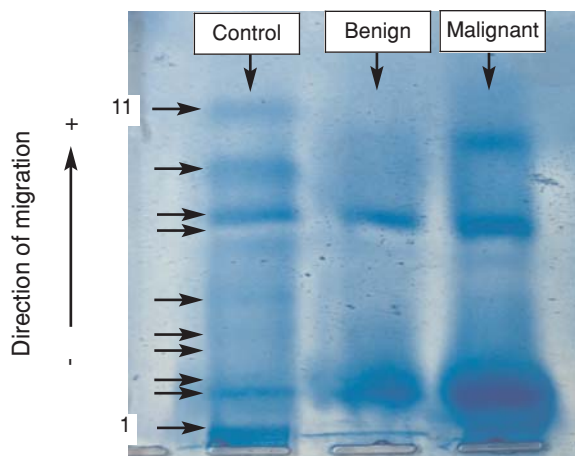
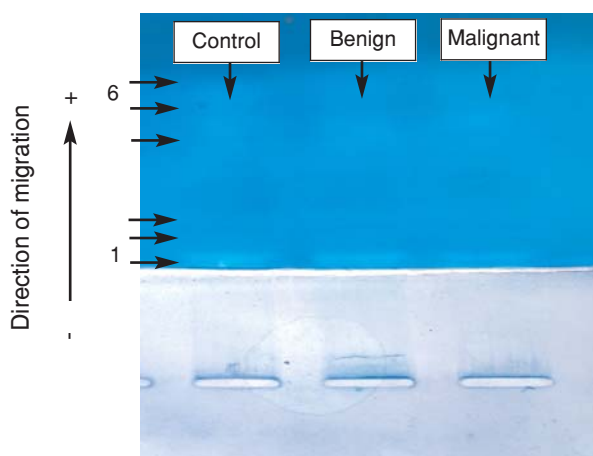


Figure 2: Conventional-PAGE electrophoresis profile of crude saliva, the gel was stained for alkaline RNase activity.



nm. The control was treated as the test sample except the sample was added after the addition of HCl (1 M) in (70%) ethanol. The activity of the enzyme was expressed in U/L and calculated as follows:

$$\text{RNase Activity } \frac{U}{L} = \frac{(\Delta A \cdot V_t \cdot \text{dilution factor})}{t \cdot (V_s)^2}$$

Where:

ΔA = Sample's absorbance - Control's absorbance

V_t = the total volume (ml)

V_s = the volume of saliva used (ml)

t = the incubation time (min)

Whereas the specific activity was expressed unit of enzyme activity per mg of protein.

Determination of Acid RNase activity in the saliva samples

The same method described above for the determination of alkaline RNase activity was used except that the pH of Davis buffer was adjusted to 5.

Conventional polyacrylamide gel electrophoresis

PAGE 12.5% technique developed by Lammeli (16), was used to analyze the crude saliva of control group and groups with breast tumors. The gel was stained for protein (17), glycoprotein (18) and RNase activity (19, 20).

Statistical analysis

The data throughout this work was reported in the form of (mean value \pm standard deviation). Quantitative differences between groups were determined by student T-test, where differences was considered as highly significant when ($p < 0.001$).

RESULTS

Ribonuclease was reported to be present in healthy human saliva in at least two different types: acid and alkaline RNase (14). In the present study acid and alkaline RNase activities were measured in the saliva samples of healthy women and women with different types of breast tumors (see above). Table 1 lists the measured protein concentration, RNase activity (using yeast RNA as a substrate) and its specific activity in the saliva of these different samples.

A highly significant increase in RNase activity and their specific activity were observed. It is clear from the results presented in the table that RNase activity and specific activity increased highly significantly in saliva samples of patients with benign ($p < 0.001$) and that of malignant breast tumors ($p < 0.001$), compared with the healthy group (Table 1). The changes in saliva protein concentration of the studied groups were similar to that of

the RNase activity, but the differences between the groups were less pronounced.

The activity of the RNase enzyme, in the saliva of women with benign tumors is more than that in healthy saliva samples in about (8.6) fold when measured at acidic pH, and (9.4) fold when the activity was measured at alkaline pH, whereas the elevation in the activity was (47.3) and (48.9) folds higher in the saliva of patients with malignant breast tumors, when measured at acidic and alkaline pH respectively, upon comparison with that of healthy individuals.

DISCUSSION

Little is actually known about human salivary RNases, though there are some reports which indicated the presence of a significant elevation in this activity in saliva of patients with different disease such as cystic fibrosis (16) and influenza (21) and ovarian tumors (22), while non significant increase was reported in the activity of salivary RNase of patient with oral tumors (23).

The elevation in RNase activities reported in present study may be due to one of the following reasons: generally it is known that cancer cells are characterized by uncontrolled increase in the number of cells and their sizes (24), which means that there will be an increase in the synthesis of different proteins and this explains the need for high RNase activity. Also cancer cells will rely on anaerobic oxidation as a source of energy by forming lactate. It was originally thought that there is hypoxia areas only in the center of tumors and remained relatively static and eventually became necrotic, while later it was known that hypoxic areas actually come and go in a tumor as perfusion varies and as new blood vessels form, fade away, and then reform (25).

Lactate acidosis has been shown to activate the biosynthesis of some acid hydrolyases, to cause release of lysosomal enzymes, to disturb calcium metabolism and change the permeability of cell membranes (26-28). Further, it has been shown that decreased intracellular pH and a decreased ATP level will cause an increase release of proteins from tissues into different body fluids (28, 29), which are in concordance with results obtained

in the present study. In addition to this the alteration in cell permeability of tumor cells membranes where a number of changes in the biochemical characteristic of malignant cell surface have been observed. These include the appearance of new surface antigen proteoglycans, glycolipids, and mucins, and altered cell-cell and cell-extracellular matrix communication (30). Such changes may lead to transport of different enzymes from the blood to the saliva via salivary glands, and one of these enzymes may be RNase. Furthermore, a transport of some activators for RNase activity such as the cations from the blood to saliva was reported to accompany the presence of some disease such as cystic fibrosis (31). Such transport may be one of the mechanisms that lead also to the observed elevation in the present study in salivary RNase activity.

Conventional electrophoresis using Lammli system in the presence of 12.5% acrylamide as a separating gel, was used, in order to detect any differences in proteins and RNase profiles. Saliva protein electrophoregrams of each group were characterized by some protein patterns typical for particular group. Saliva of the first group (control) contained up to 11 protein bands different in its molecular mass, while electrophoresis of saliva samples of the second group (benign) was characterized by significant reduction of proteins bands (about 6 protein bands). But in the malignant group (third group) the electrophoresis patterns of saliva proteins revealed up to 7 proteins, figure (1). This can be explained by the deletion hypothesis of cancer proposed by Miller (32), which suggested that carcinogenesis resulted from a permanent alteration or loss of protein essential for the control of growth. Potter (33) suggested that the proteins lost during carcinogenesis may be involved in the feedback control of enzymes systems required for cell division. There were insignificant variations of proteins bands within each group, but they did not affect the characteristic pattern of saliva proteins content typical for these groups. The electrophoresis pattern of saliva samples for the three studied groups, which were stained for RNase activity, is shown in figure (2). Six activity bands appeared in the saliva of each group, four of them traveled in the gel to the same

distance, while the other two traveled with different mobility. Band number 5 in the saliva of the control group seems to move further than that present in the saliva of the benign and malignant groups, while band number 6 in the control group moves slower distance than the same band in the other two groups.

CONCLUSION

Out of the result of the present study, RNase seems to be

a promising parameter that may be used to differentiate the presence of malignancy. Further study is carrying out in our laboratory to check the selectivity of this biochemical marker.

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