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DETECTION OF CIRCULATING AUTOANTIBODIES TO HA-RECEPTORS IN HUMAN LUNG CANCER

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ABSTRACT

Identification of circulating autoantibodies to hyaluronan-receptors in lung cancer holds great promise and these HA-receptors might be a potential biomarker, but remains technically challenging. Interaction and involvement of hyaluronan with specific cell surface receptors such as CD44, RHAMM and with intracellular HABP in modulating cellular behavior has been predicted. In the present study, we detected autoantibodies and their corresponding antigens (HA-receptors) by immunoblotting and sensitive ELISA. Identified antigens (HA-receptors by immunoaffinity purified autoantibody IgM) whether have binding motifs to HA or not, were further confirmed by competition experiments. HA abolish 91% binding of autoantibodies to it's receptors. These results implicate that autoantibody IgM reacting proteins might be a HABPs. In conclusion, identification of circulating tumor antigens (HA-receptors) or their related autoantibodies provide a means for early detection and diagnosis of cancer.

KEY WORDS: **circulating autoantibodies. HA-receptors, hyaluronic acid, lung cancer, biomarker**

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INTRODUCTION

Lung cancer is the most common and lethal malignancy in the world with 1.3 million new cases diagnosed annually and over 1 million deaths reported each year[1,2]. Tobacco smoking is estimated to cause around 90% of all cases in men and 80% in women[3]. However, other recognized risk factors for lung cancer include passive smoking; occupational exposures especially to asbestos; and radon[2]. The latency period for lung cancers attributable to smoking is at least 20 years[2]. Lung cancer is often detected by a chest x ray examination, by which time it is usually advanced and patients cannot be cured. Currently there is no accepted early diagnostic test, although screening trials using spiral CT in people ''at risk'' is the only choice[4.] Circulating autoantibodies elicited by the patient's own immune system after exposure to cancer proteins are emerging as promising biomarkers for the early detection of cancer. An advantage of autoantibodies as biomarkers is their production in large quantities despite the presence of a relatively small amount of corresponding antigen. Identification of circulating tumor antigens (HA-receptors) or their related autoantibodies provides a means for early detection and diagnosis of cancer. During tumor progression HA-receptors are over expressed in cancer and transformed cells, but show little expression in normal differentiated cells. Many HA-receptors such as CD44, RHAMM, P-32, and TSG- 6 have been implicated in human carcinogenesis using the antibodies that were generated against these proteins[5-8]. Among these HA-receptors, CD44 has been studied very well in various types of human cancers, and its expression is correlated with a favorable prognosis in some cancers[9-11]. But unfavorable in others[12,13]. Other HABPs have been studied only in a limited number of cancers. Given the differential expression of the known HABPs in human carcinogenesis, the possibility of common HA-receptor involved during human tumor progression is still speculative. Although human antibody response to HA-receptors has not yet been described in lung cancer patients, we examined prevalence of autoantibodies to HA-receptors in lung cancer patients with a specific ELISA using human circulating antigen. In accordance with this in the present study an attempt was made to detect autoantibodies to HA-receptors in lung cancer patient's serum and which can be used in the early detection and diagnosis of cancer.

MATERIALS AND METHODS

We tested total of 40 serum samples from patients with lung cancer (n=25, mean age ±SD=50.6±14.4 years and normal control groups (n=15, mean ±SD=38. ±15.5 years) All human samples were collected prospectively at Preethi center for oncology. The sera used for this detection of autoantibody to HA-receptors by ELISA assay were from individual Institutional Ethical Review Board–approved, patients consent was taken. The blood samples were centrifuged at 1250g for 15 min, and aliquot of sera were stored at -80^0 C until use. The serum samples were thawed only once before use, and these diluted serum samples were never used again.

Detection of autoantibodies to HA-receptors by ELISA

Circulating antigen was used in this study, briefly, circulating autoantibodies to HA-receptors in the sera of patients with lung cancer and healthy individuals were measured by solid phase ELISA assay. For such assay, Maxisorp flat-round bottom high protein-binding capacity polystyrene (Nunc Denmark) 96 well plates were coated with 100µl of diluted (5µg/mL concentration with 50mM Bicarbonate buffer pH 9.6) sera from lung cancer and healthy individuals. The plates were incubated at room temperature for 1h, then washed couple of times with washing buffer (PBS-Tween 20 pH 7.4) the non specific binding sites were blocked with 100µl of 5% non fat dry milk and 1% bovine serum albumin [BSA], pH 7.0) for 2 h at room temperature, then the plates were washed twice with sodium citrate washing solution (50mM sodium citrate ,0.15M sodium chloride, 0.1% polyoxyethylenesorbitan monoluarate tween 20 pH 7.4), the serum samples are diluted to 100-25000 fold with samples dilution buffer PBS pH 7.4 1% BSA,

0.05% Tween 20 and 100µl of diluted samples are added to each well, then incubated for 1hour at 37^0C. After three washes with the sodium citrate washing solution ,100ul of 15000 fold diluted goat anti-human HRP antibody enzyme conjugate (Biolegend USA) in PBS containing 1% BSA was added to the wells, after which the plates were incubated for 1h at room temperature. After washing the plates five times in sodium citrate washing solution,100µl of ABTS (2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid Tokyo chemical industry co., Ltd), in 0.1M Na_2 HPO_4, and 0.1M citrate buffer pH 4.0 with 30%H_2O_2, Was added. The reaction was stopped with 100µl of 0.2M sodium citrate buffer after which the absorbance read at 450nm was recorded on an ELISA reader (TICAN Sweden)[14]

Preparation of biotinylated hyaluronic acid probe

500µg of the hyaluronic acid (across USA) was dissolved in 500µL of 0.2 M MES buffer (pH 5.5). To this solution, 1mM biotin-LC-hydrazide (dissolved in DMSO) and 10mM EDC were added. The reaction mixture was incubated at 4ºC for 16hrs. This was dialyzed against PBS pH 7.4 for 36hrs at 4ºC. Finally, the dialyzed bHA was stored in glycerol at –20ºC[15].

Detection of HA-receptors using bHA polymer probe

Equivalent amounts of protein (50 µg) from normal and lung cancer serum samples were separated under reducing conditions on 10%SDS-PAGE at 25 mA constant current and transblotted onto PVDF membrane by electroblotting [wet transfer] at 200 mA current for 1hr. After transblotting, the membrane was washed with T-TBS pH 8.0 (Tris tween-20 buffer saline) and non-specific reaction were blocked with blocking buffer for 1hr at room temperature. The membrane was extensively washed with T-TBS followed by incubation with 1:100 dilution of bHA probe for 1hr at room temperature and then incubated overnight at 4^0C. The membrane was washed with T-TBS. The membrane was treated with HPO-9 (1:20,000) for 45 min. The membrane was washed couple of times with T-TBS. The immune reactive proteins (hyaluronic acid binding proteins) were visualized with ECL detection system (Amershan)

Isolation and purification of human circulating autoantibody (IgM)

A saturated solution of ammonium sulphate pH 7.4 (with ammonium hydroxide) was prepared and added to serum samples with stirring to achieve a 50% saturated solution and this was allowed to mix in vortex for 1hr at 4 ^{0} C. Later mixture was centrifuged at 10,000g for 20 min at 4 ^{0} C and pellet was resuspended in 100mM Tris containing 150mM NaCl (pH 8.0), and was dialyzed overnight against same buffer at 4^0 C. Sephacryl S-300 column was equilibrated in phosphate buffer pH 7.4 and the flow rate was adjusted to 15 mL per 1hr. Serum samples were subjected onto column and elution was carried out with 100mM Tris containing 150mM NaCl pH 8.0 and all fractions were read at 280 nm, and each fraction was pooled dialyzed and lyophilized. Further, affinity chromatography was carried out to obtain highly purified antibody as described below.

Affinity purification of IgM autoantibody using Anti-IgM antibody conjugated CNBr activated Sepharose 4B

Anti- human IgM (µ-chain specific) affinity purified antibody (KPL Biolegend USA) was dialyzed against coupling buffer (50mM Carbonate/bicarbonate buffer pH 9.6) for 24hrs with 3 changes. The dialyzed antibody is allowed to conjugate with CNBr-activated Sepharose 4B (GE Healthcare Sweden) and allowed to mix overnight for 16hrs at 4^0 C. The affinity matrix was loaded onto a glass column (0.5 cm × 2.0 mL bed volume) and packed at a flow rate of 1.5 mL/min using 10mM Tris pH 8.0 as mobile phase. The mixture was packed on to a 1.0mL column. The unbound antibody was washed using coupling buffer. In order to block the free reaction groups, the column was preequilibrated with 0.5M glycine (50mM final concentration).Then the column was again washed and equilibrated with 50mM Tris pH 8.0. To the above the column, Sephacryl S-300 size exclusion chromatography pooled peak-I was subjected to anti-IgM affinity chromatography after collecting unbound material the column was washed with 10mM Tris pH 8.0 to remove the unbound protein. Bound protein was eluted

with 50mM glycine HCl, pH 2.5, and the protein rich fractions were immediately neutralized, by adding 1M Tris pH 8.5; Pooled fractions were dialyzed against 10mM Tris pH 7.4; The volume of the concentrated solution was then reduced by Centricon filter units of 10000 MWCO (Millipore, Nepean Canada), purity was determined by UV spectrophotometer and ouchterlony double diffusion, and quantification of total protein was accomplished by bradford assay, the IgM content and immune reactivity were detected by ELISA[16]

Detection of autoantibody-IgM specific receptors

50µg of serum samples were subjected to 10% SDS-PAGE, under reducing conditions at constant voltage (25mA) and electro blotted onto PVDF membrane (Millipore USA) 45min at 200 mA then membrane was blocked with 5% inactivated human serum,1% BSA, 5% non fat dry milk (Sigma) for 1hr at room temperature and the membrane was washed with T-TBS pH 8.0) and the blot strips were incubated with patients cancer and normal sera 1:100 fold diluted in 0.05% of Tween 20 T-TBS pH 8.0 overnight at 4^0 C and the strips were washed, then incubated with goat anti-human IgM-horseradish peroxidase conjugate (10,000 fold dilution), for 1hr at room temperature and immune reaction was monitored by enhanced chemiluminescence (Amersham Pharmacia).

Competitive ELISA with HA

For competitive Enzyme Linked-Immunosorbent Assay (ELISA), 5µg/mL circulating antigen was diluted in 50mM carbonate/bicarbonate buffer pH 9.6. and 100µL of samples were dispensed to 96 wells polystyrene flat bottom microtiter plates (nunc, Denmark) and incubated for 1hr at room temperature. Plates were washed thrice with PBS-T pH 7.4 (Tween-20 phosphate buffer saline) and the unoccupied sites were blocked by 100µL of 5% $CaCl_2$ heat inactivated human serum 1% BSA in PBS-T for 1hr at room temperature. The plates were washed and incubated with 100µL of unlabeled HA (5µg/mL) overnight at 4^0 c and washed thrice and re incubated with HA and 1µg of IgM antibody 1hr at room temperature After washing, the plates were incubated with 100µL of goat anti-human HRP conjugate. The reaction was developed with ABTS and the reaction was stopped with 0.2M citric acid, read at 450nm and the absorbance was measured as mean ± SD% of competition. The above result have been confirmed by western blot, equivalent quantities of circulating antigen (50µg) from normal and lung cancer samples were taken in duplicate and boiled with sample buffer for 5 min. The protein samples were separated under reducing conditions on 10% SDS-PAGE at 25 mA constant current and transblotted onto PVDF membrane by electroblotting [wet transfer] at 200 mA for 1hr. After transblotting, the membrane was washed with T-TBS-pH 8.0 and blocked with blocking buffer for 1hr at room temperature. One set of transblotted proteins was reacted with IgM antibody. The other set of transblotted proteins was incubated with 500 µg of non-biotinylated HA for 1hr at room temperature, followed by incubation overnight at 4^0 C with mixture of 1 µg of IgM antibody and 500 µg of non-biotinylated HA. The membrane was washed with TTBS and was treated with goat ant-human HRP conjugate (1:10,000) for 45 min. It was washed again for a couple of times with TTBS and the immune reactive proteins were detected using ECL detection system.

STATISTICAL ANALYSIS

Statistically significant differences between the groups were determined by student t-test and expressed as mean ± standard deviation. P-value of < 0.05 was used to determine statistical significance. All analyses were performed using graph pad prism 5.0 software

RESULTS

In the present investigation, an attempt has been made to detect autoantibodies to HA-receptors, in the sera of patients with lung cancer and normal serum. A total of 25 patients with lung cancer and 15 healthy individuals were analyzed. The presence of HA-receptors autoantibodies in the serum was examined using ELISA and western blott analysis in which circulating lung cancer antigen was immobilized and incubated with patient and normal sera (Fig.1) autoantibodies to HA-receptors were detected

in the sera of patients with lung cancer. Though they were detected in healthy individuals it was not significant when compared to cancer patients. Difference was statistically significant $p < 0.001$.

To determine whether circulating lung cancer antigen has hyaluronic acid binding region, the samples were run under reducing 10% SDS-PAGE and transblotted onto PVDF membrane and reacted with bHA probe (Fig.2).This experiment justifies aforesaid conclusions. (Fig. 2).

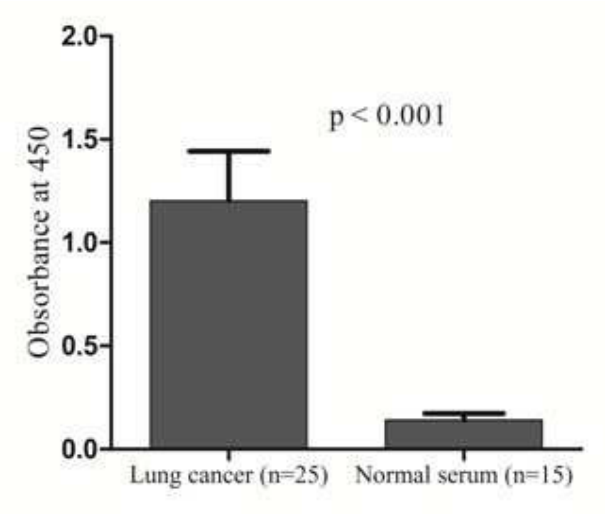

Figure 1

Figure.1 Levels of HA-receptors autoantibodies in sera from patients from lung cancer and normal individuals were detected by ELISA. Levels of HABPs autoantibodies significantly elevated in patients with lung cancer compared to healthy individuals (statistical significance p < 0.05).

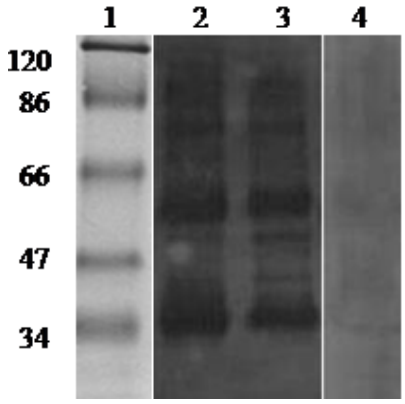

Figure 2

Western blot analysis to detect HA polymer binding proteins using bHA as a probe; western blot was performed to detect the HA-receptors from patients with lung cancer (lane 1 molecular weight markers, lane 2-3 lung cancer serum and lane 4 healthy individuals by trasblotting 50µg of crude proteins and incubating the blot overnight at 4^0 C with bHA polymer, then reacted with HPO9 and developed with ECL. bHA probe detecting over expressed multiple hyaluronic acid binding proteins in lung cancer but no reaction was seen in healthy individuals.

To characterize circulating IgM, serum from lung cancer and normal subjects were precipitated by 100% saturated ammonium sulphate final 50% then after dialyzed and concentrated by Centricon tubes, and subjected to Sephacryl S-300 gel filtration

analysis and the eluted fraction was tested for IgM activity, I peak with a strong reactivity was observed for HABPs, thus providing first evidence of the occurrence of circulating IgM in the patients sera. We observed significantly elevated level of the immunological marker IgM in lung cancer compared to control sera. [Fig.3]. In order to purify circulating autoantibodies IgM from sera of patient with lung cancer sera, I peak of sephacryl S-300 was added on anti-IgM affinity column and then characterized on native and SDS-PAGE, followed by immunoblotting and ELISA. To confirm the specificity of these autoantibodies to HA-receptors ELISA was used as a tool. Immune affinity purified IgM reacting to circulating HABPs in the sera of patient with lung cancer as well as in the sera of normal individuals. To assess the relevance of this finding, IgM autoantibody was strongly reacted with HA- receptors in patients with lung cancer compared to healthy individuals (Fig.4) and this above results have been confirmed by HA competition experiments.

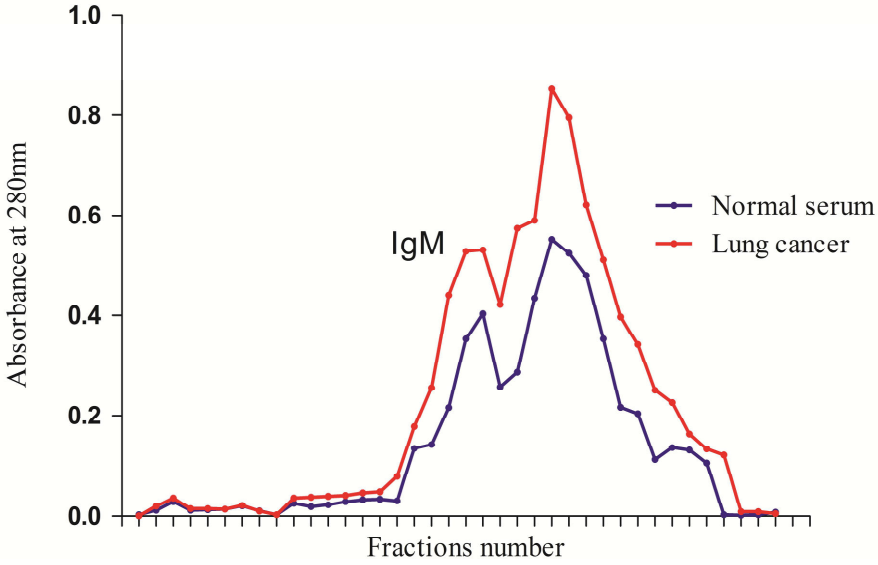

Figure 3

Purification of circulating IgM by gel filtration chromatography. Elution profile of protein monitored at an absorbance of 280 nm. After ammonium sulphate precipitation, the concentrated sample was subjected on sephacryl S-300 size-exclusion column with 100mM Tris, 150mM NaCl pH 8.0, at a flow rate of 15-18 mL/ h.

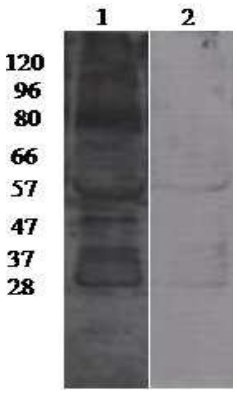

Figure 4

immune blotting analyses of human circulating HA-receptors were detected by affinity purified IgM antibody (lane-1 lung cancer) (lane-2 normal serum). The circulating antigen was subjected to SDS PAGE, and transferred onto PVDF. The membrane was incubated with affinity purified IgM and followed by ECL. This circulating antigen which was detected by IgM antibody strongly reacted in lung cancer patients but not in healthy individuals. And this result hand in glove with previously characterized HA-receptors (120, 80, 57, 37-kD) as a major proteins.

The specificity of autoantibody IgM to the HA-receptors was asserted by competitive inhibition studies. HA Competition analysis was conducted by ELISA and western blotting. Since HA has already bound to the binding protein. These results were shown (Fig.5A & 5B), the HA 91% blocked binding of the autoantibodies to HA-receptors in lung cancer and normal serum. Therefore, the autoantibodies reacting proteins might be a HABPs.

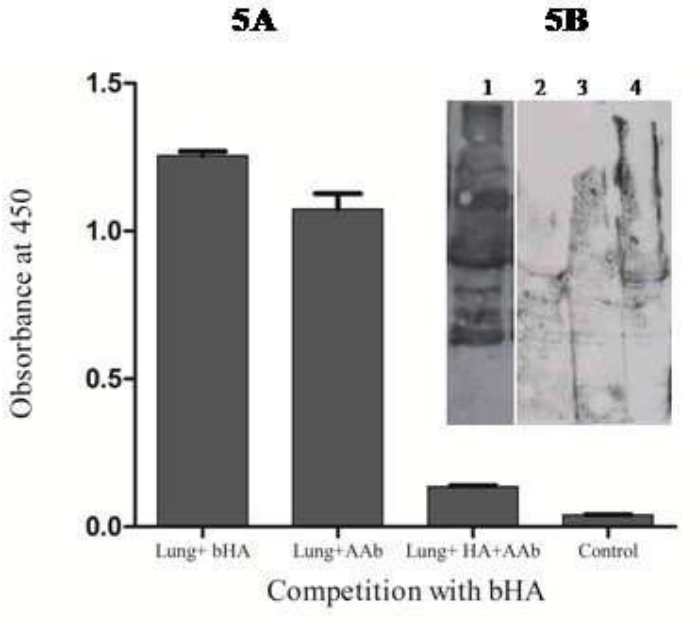

Figure 5A & 5B

Competition analysis between HA and affinity purified IgM antibody: Competition analysis was conducted when circulating antigen was bound to the maxisorp polystyrene plate's wells and they are subsequently exposed to known concentration of unlabeled HA, (Fig.5A) And the results have also been proved by western blotting, (Fig 5B) circulating antigen reacted with IgM showed strong reaction at 120, 80, 57, 37kDa, (lanes 1), whereas, the blots incubated with the HA completely abolished IgM binding to HA-receptors (lane 2-4).

DISCUSSION

The development of circulating autoantibodies to tumor associated antigen (TAA) has been observed to be associated with cancer[17,18]. In this study, sera from patients with lung cancer exhibited IgM autoantibodies to HA-receptors. Identification of cancer earlier require the early identification of cancer biomarker, HA-receptors might be a potential biomarker, these over expressed HA-receptors have been implicated in tumorigenesis[19,20] but their involvement varies. The interaction of hyaluronan with specific cell surface receptors such as CD44, RHAMM and with intracellular HABP in modulating cellular behavior have been predicted[20,21]. The speculation that HABP is involved in tumorigenesis is substantiated with the several findings suggest that HA receptors (HABP) play an important role in tumor metastasis. Recently, the identification of hyaluronic acid binding proteins such as CDC37[22] RHAMM/IHABP[5,6] and P32 [7] and IHABP4[8] evoke an interesting questions. Several autoantibodies have been identified in lung cancer and propose as potential serum diagnosis markers Annexins I and II[23] p[53 24] NY-ESO-1[25]. livin and survivin[26] Among them, p53 autoantibodies in lung cancer has been largely investigated and their prevalence observed approximately 30% of patients with lung cancer[27]. Interestingly, the presence of p53 autoantibodies is highly correlated with p53 gene missense mutations and accumulation of p53 in the tumor (60–70%). Remarkably, p53 autoantibodies appear very early in carcinogenesis, even before the cancer diagnosis[28,29]. Recently, SOX

autoantibodies have been shown to have diagnostic relevance in discriminating early-stage lung cancer detection[30]. In the present investigation we have detected autoantibody to HA-receptors in lung cancer. And we had found their level was significantly elevated. In order to validate, autoantibodies reacting proteins whether have similar binding motifs for HA or not, we have prepared unlabeled HA and labled HA (bHA probe) we had found autoantibody IgM reacting proteins might also belongs to be family of HABPs. The recognition of the HA-receptors by IgM antibody, and the specificity of IgM autoantibody to HA-receptors were validated by competitive inhibition studies. Interestingly, HA exhibit 91% Competition of binding of autoantibodies to HA receptors, since HA have already bound to the binding protein. These results implicate that autoantibody IgM reacting proteins might be a HABPs.

CONCLUSION

In the present study, we detected elevated level of autoantibodies IgM and their corresponding antigens (HA-receptors) in lung cancer. At present there is little to offer for early diagnosis, even in those at high risk of developing the disease. Autoantibodies have been shown to be present in the circulation of people with various forms of malignancy before cancer-associated antigens can be detected, and these molecules can be measured up to 5 years before symptomatic disease. therefore, detection of autoantibodies to a panel of HA-receptors might have potential to provide clinicians with the opportunity to detect early amplification of the carcinogenic signal, thereby might providing a sensitive, specific and simple screening tool for the early diagnosis and subsequent early clinical intervention of lung cancer. Further studies are under prospective to validate and characterize a specific protein in different types of tumor, and determine their homology with known HA-receptors.

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