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Scientific Research

Angiotensin-(1-7) Changes Apoptosis-Related Genes Expression in Human Breast Cancer Cell Line T47D

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Abstract

Angiotensin-(1-7) [Ang-(1-7)] is a heptapeptide of the renin-angiotensin system with vasodilator and anti-proliferative properties. In the present study, we aim to investigate whether Ang-(1-7) induces apoptosis in breast cancer cells and whether the altered expression of apoptosis-related genes is involved in this process. Human breast cell line T47D was treated with angiotensin-(1-7) and angiotensin II (Ang II). Cell proliferation and apoptosis were quantified using hemocytometer and flow cytometry, respectively. The expression of 84 apoptosis-related genes was evaluated through qPCR array. Ang-(1-7), as opposed to Ang II, decreased proliferation and increased apoptosis in T47D cells. Moreover, many pro-apoptotic genes were up-regulated, such as *BAK*1, *BAX*, *BCL2L*1, *BID* and *BIK.* In addition, some anti-apoptotic genes as *AKT*1 and *XIAP* were down-regulated by heptapeptide. Although a deeper study should be performed, our results support the hypothesis that Ang-(1-7) could change the expression of several genes related to apoptosis, interfering directly in the molecular pathways associated with the survival of breast cancer cells.

Keywords

Angiotensin-(1-7), Breast Cancer Cells, Apoptosis, qPCR Array

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1. Introduction

Breast cancer is the most common cancer in women and the second leading cause of cancer death in women worldwide [1] [2]. It is a multifactorial disease caused by epigenetic changes and genetic mutations that occur in genes directly involved in the process of cell division and programmed cell death [3]-[5]. Therefore, studies on chemotherapy agents and their mechanisms of action are particularly relevant.

Studies have shown that the components of the renin-angiotensin system (RAS) act in the carcinogenic process [6]-[8]. Angiotensin II (Ang II), considered the main effector of the RAS, has vasoconstrictive, angiogenic and proliferative properties [9]-[12]. Angiotensin-(1-7) [Ang-(1-7)] is an endogenous 7-amino acid peptide hormone of the RAS that has vasodilator, anti-proliferative and anti-angiogenic actions [13]-[17]. Thus, Ang-(1-7) opposes the action of Ang II.

The relationship between genetic polymorphisms of the Angiotensin II, Angiotensin-(1-7) and Angiotensin-Converting Enzyme (ACE) with breast cancer was demonstrated in many works [18]-[21]. *In vitro* assays show that Ang-(1-7) inhibits the proliferation of lung cancer cells [22]. Ang-(1-7) treatment decreased microvessel density associated with a reduction in both vascular endothelial growth factor and placental growth factor in lung and breast tumor xenograft [23] [24]. It has also been demonstrated that Ang-(1-7) inhibits tumoral fibrosis [25] and attenuates multilineage cytopenias following chemotherapy [26]. All these results suggest that heptapeptide might be a general regulator of cell growth, suggesting its potential use as a promising antitumor agent.

Programmed cell death (apoptosis) is considered an innate defense mechanism leading the cell to become antineoplastic; similarly, many chemotherapeutic agents act by inducing this type of cell death [27]. Therefore, we aim to investigate whether Ang-(1-7) induces apoptosis in breast cancer cells and whether the altered expression of apoptosis-related genes is involved in this process.

2. Materials and Methods

2.1. Cell Culture and Treatments

The human ductal breast epithelial tumor cell line T47D was originally obtained from ATCC (American Type Culture Collection). T47D cells was grown in DMEM supplemented with 10% FBS, 100 UI/mL penicillin, 100 ug/mL streptomycin (all from Invitrogen), 2 UI/mL human insulin, and 4 mM glutamine (both from Sigma-Aldrich). The control group was treated with DMEM supplemented only. The experimental groups were treated with DMEM supplemented and peptides: Ang II and Ang-(1-7) in the concentration of 10^{-6} M. The peptides were replaced daily due to their rapid degradation [28].

2.2. Cell Proliferation Assay

T47D cells were seeded into 24-well plates (1×10^3 cells/well). After 2, 6, 9, and 15 days the cells were removed from triplicate wells using trypsin/EDTA and counted using a hemocytometer.

2.3. Apoptosis Assay

T47D cells were seeded into 24-well plates. After 2, 6, 9, and 15 days of treatment the cells were removed from triplicate wells using trypsin/EDTA. Posteriorly, cells were stained with Annexin V using the Guava Nexin kit (Millipore) according to the manufacturer's instructions. The cells from each well were resuspended in 100 µl of supplemented DMEM (1×10^5 cells/well) and 100 µl of the Guava Nexin Reagent. Samples were incubated in the dark at room temperature for 20 min and subsequently evaluated in the Guava easyCyte™ cytometer (Millipore).

2.4. RNA Extraction

Total RNA was extracted from T47D cells (treated with peptides for 48 hours) using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions, and subjected to DNase treatment using an RNase-free DNase kit (Qiagen). The quantity and quality of extracted RNA were measured by spectrophotometer (NanoDrop Technologies).

2.5. qPCR Array

The Human Apoptosis RT2 Profiler PCR Array (SA Bioscience) was used to simultaneously evaluate the ex-

pression profile of 84 key genes involved in programmed cell death.

Total RNA (2 μg) was converted to cDNA via reverse transcriptase reaction using the First Strand Kit (SABiosciences), according to the manufacturer's instructions. This kit includes a proprietary buffer that eliminates residual genomic DNA contamination in cell line RNA samples. A reaction mix containing cDNA and all the optimized reagents and buffers needed for SYBR® Green were prepared based upon real-time polymerase chain reactions. Twenty-five microliters of this mix was added to each well of the 96-well plate, which was then loaded out on a 7500 Fast Real-Time PCR System (Applied Biosystems) and run in a two-step cycling program with one initial cycle of 10 min at 95˚C and 40 cycles of 15 s at 95˚C, and one final cycle of 1 min at 60˚C, in a 7500 Fast Real-Time PCR System (Applied Biosystems).

2.6. Statistical Analysis

Cell proliferation and apoptosis assays were analyzed by descriptive statistics (means and standard deviation) and inferential statistics through the Student's *t*-test with significance level of 5% ($p < 0.05$). qPCR Array reactions were analyzed trough the RT2 Profiler™ PCR Array Data Analysis software (SABiosciences). We only considered p values below 0.05 and fold regulation values higher than 2.00 to be statistically significant.

3. Results

3.1. Cell Proliferation Analysis

Ang-(1-7) decreases the proliferation of T47D cells, while Ang II increased the proliferation of these cells (**Figure 1**). Ang (1-7) had opposite effects to Ang II.

3.2. Apoptosis Analysis

Ang-(1-7) increased apoptosis in T47D cells after 6, 9 and 15 days (**Figure 2**). The apoptotic effect on breast cancer cells was similar in all treatment periods (except 2 days) After 2 days of treatment we observed a high percentage of apoptotic cells, but there were no significant differences between groups.

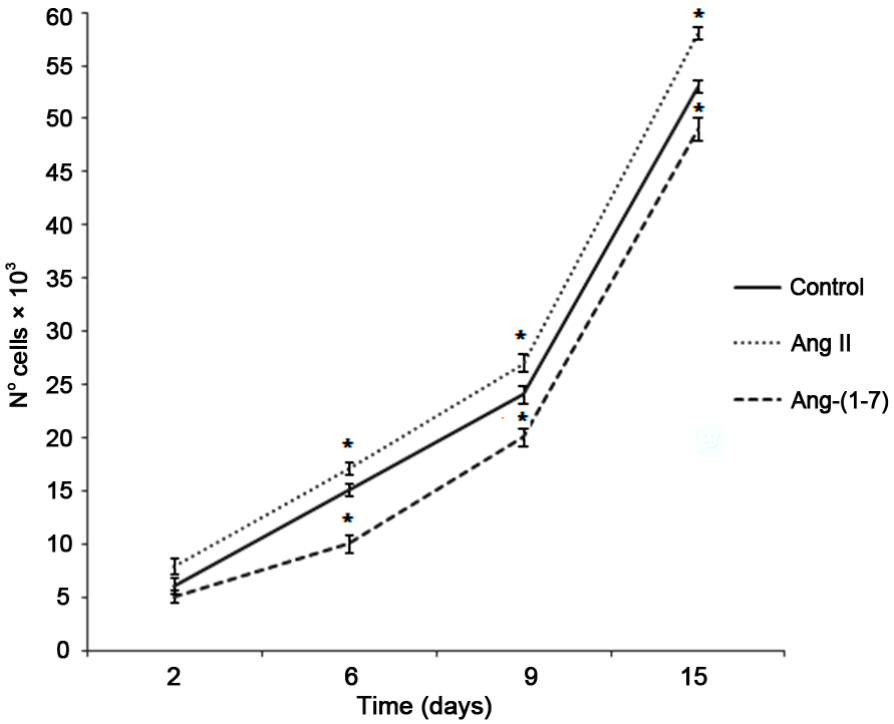

Figure 1. Proliferation of T47D cells after treatment with Ang-(1-7) and Ang II. *$p < 0.05$ (compared to control).

3.3. qPCR Array Analysis

The qPCR Array assay was performed to determine the expression profiles of 84 apoptosis-related genes. Ang-(1-7) altered the expression of 60 genes compared to the control group; 47 were up-regulated and 13 were down-regulated, whereas Ang II altered the expression of 22 genes; 7 were up-regulated and 15 were down-regulated (**Table 1**).

Some anti-apoptotic genes were down-regulated due Ang-(1-7) treatment (**Figure 3**). In contrast, several genes involved in the induction of apoptosis were up-regulated by heptapeptide (**Figure 4**). Most of the times, Ang II had opposite effects to Ang-(1-7).

4. Discussion

In the present study we investigated the pro-apoptotic effects of the Ang-(1-7) in human ductal breast epithelial tumor cell line T47D. This line was selected specially for display luminal phenotype with positive expression of estrogen and progesterone, thus constitutes an experimental model which represents approximately 75% of cases of breast cancer diagnosed in clinical practice.

Our results show that Ang-(1-7) hormone has anti-proliferative and pro-apoptotic actions on T47D breast cancer cells. A similar effect was evidenced in studies with human prostate malignant cells [29] and human lung cancer cells [22] [24].

Ang-(1-7) inhibited the proliferation of T47D breast cancer cells, whereas Ang II favored the proliferation of these cells. Other studies conducted in mammary cells showed similar results [30]-[32]. Our findings therefore further confirm that Ang-(1-7) and Ang II often have opposing biological actions.

Analysis of apoptosis by flow cytometry showed that Ang-(1-7) is able to induce apoptosis in breast tumor cell line, but does not cause this effect in normal mammary epithelial cell line MCF-10A. In addition, the qPCR assay performed in T47D cells demonstrated that the Ang-(1-7) might change the expression of more than 70% of apoptosis-related genes (55.9% genes were up-regulated and 15.5% were down-regulated). On the other hand, Ang II changed the expression of 26% of apoptosis-related genes (8.3% genes up-regulated and 17.9% genes down-regulated).

Apoptosis is induced via two main routes involving either the mitochondria (the intrinsic pathway) or the activation of death receptors (the extrinsic pathway). The intrinsic pathway is induced by various types of intracellular stress and is mediated by members of the *BCL*-2 family. The extrinsic signaling pathway involves the activation of transmembrane death receptors that are members of the TNF superfamily. Both pathways converge to induce the activation of initiator and executioner caspases, leading in programmed cell death [33] [34].

The expression of caspases 1, 2, 4, 5, 7, 9, 10 and 14 was increased after treatment with Ang-(1-7). The association of these caspases with apoptosis has been described in several studies [35]-[41].

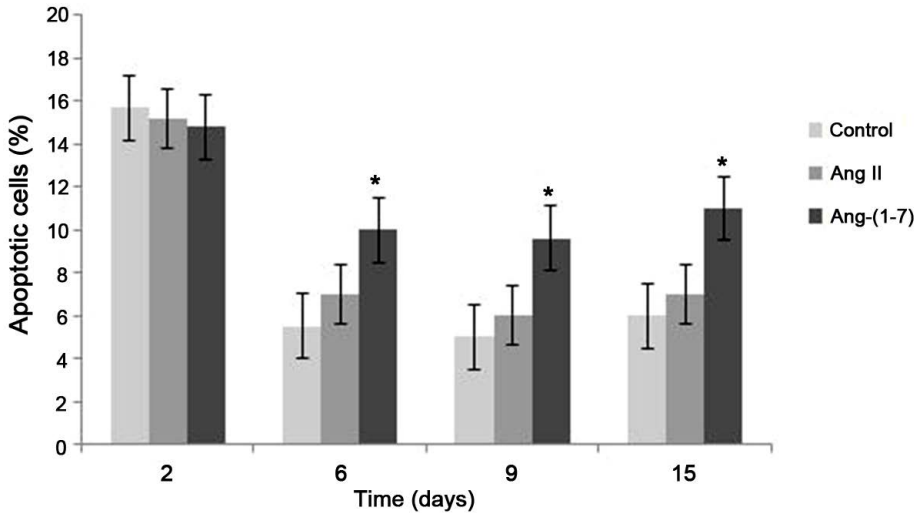

Figure 2. Apoptosis percentage of T47D cells after treatment with Ang-(1-7) and Ang II. $^{*}p < 0.05$ (compared to control).

Table 1. Apoptosis-related genes expression profile in human breast cancer cells after Ang-(1-7) and Ang II treatment.

Gene Symbol	Fold change Ang-(1-7) × CT	Fold change Ang-II × CT	Gene Symbol	Fold change Ang-(1-7) × CT	Fold change Ang II × CT
ABL1	14.5827	0.8335	CASP7	3.5602	0.831
AKT1	0.4335	0.422	CASP8	0.5605	0.8348
APAF1	1.2685	0.836	CASP9	3.8289	1.6668
BAD	1.6643	0.8292	CD40	2.9917	2.0927
BAG1	1.2078	0.8423	CD40LG	9.5977	0.8327
BAG3	0.8741	0.8415	CFLAR	5.4723	1.7056
BAG4	0.2102	0.8399	CIDEA	9.5977	3.5808
BAK1	2.604	1.6774	CIDEB	1.4202	0.4142
BAX	9.3295	0.4154	CRADD	0.9825	1.6924
BCL10	0.1657	1.6664	DAPK1	9.5977	0.8327
BCL2	14.7275	1.6287	DFFA	0.1422	0.8319
BCL2A1	0.2139	1.6847	FADD	0.4021	0.4185
BCL2L1	10.5406	0.4134	FAS	0.3109	0.4174
BCL2L10	9.5977	0.8493	FASLG	9.5977	0.8327
BCL2L11	0.8152	1.6665	GADD45A	2.2975	0.4233
BCL2L2	1.1388	1.6713	HRK	0.9564	1.6665
BCLAF1	0.1145	1.6424	IGF1R	1.4168	3.3446
BFAR	0.1481	1.6861	LTA	9.5977	0.8879
BID	2.2737	0.8362	LTBR	9.4426	0.8435
BIK	4.5455	1.3173	MCL1	10.1351	3.3414
NAIP	2.345	0.8453	NOL3	0.7898	1.6754
BIRC2	0.8614	1.6483	PYCARD	3.2496	0.8455
BIRC3	0.8803	0.8456	RIPK2	3.0304	1.6477
XIAP	0.2851	0.8333	TNF	19.5397	3.3521
BIRC6	2.862	1.69	TNFRSF10A	0.9505	0.2116
BIRC8	9.5977	3.5536	TNFRSF10B	0.6384	0.8365
BNIP1	0.3422	0.8315	TNFRSF11B	9.5977	0.8327
BNIP2	3.2399	0.8434	TNFRSF1A	5.4577	1.6898
BNIP3	0.6969	1.6738	TNFRSF21	1.0109	0.8427
BNIP3L	1.8115	1.6647	TNFRSF25	1.1189	0.4218
BRAF	2.5251	1.6606	CD27	9.048	1.0456
NOD1	2.4816	1.6586	TNFRSF9	9.5977	0.8708
CARD6	1.115	0.4216	TNFSF10	5.1556	0.8337
CARD8	0.5609	1.0618	CD70	9.5977	2.8259
CASP1	2.3419	1.6759	TNFSF8	9.5977	0.8327
CASP10	9.5977	0.8582	TP53	0.2649	0.6586
CASP14	8.9969	0.7806	TP53BP2	0.1871	0.8422
CASP2	7.9715	0.4181	TP73	2.2379	1.6741
CASP3	1.1176	1.6624	TRADD	2.2328	0.8334
CASP4	5.3219	0.4193	TRAF2	3.0765	0.42
CASP5	9.5977	0.8327	TRAF3	7.3658	0.8305
CASP6	1.7785	0.4178	TRAF4	3.6426	0.2059

Fold-change and fold-regulation values greater than 2 are indicated in red, fold-change values smaller than 0.05, and fold-regulation values smaller than −2 are indicated in blue.

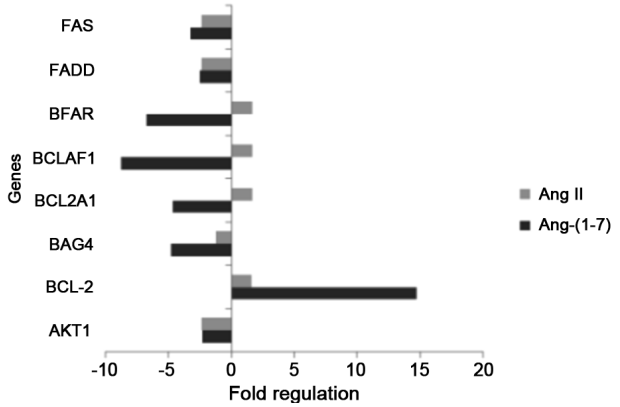

Figure 3. Anti-apoptotic genes with altered expression after Ang-(1-7) and Ang II treatment. T47D cells were treated for 48 hours with the peptides and gene expression was verified by qPCR. Fold-regulation values greater than 2 indicate up-regulation and fold-regulation values smaller than −2 indicate down-regulation.

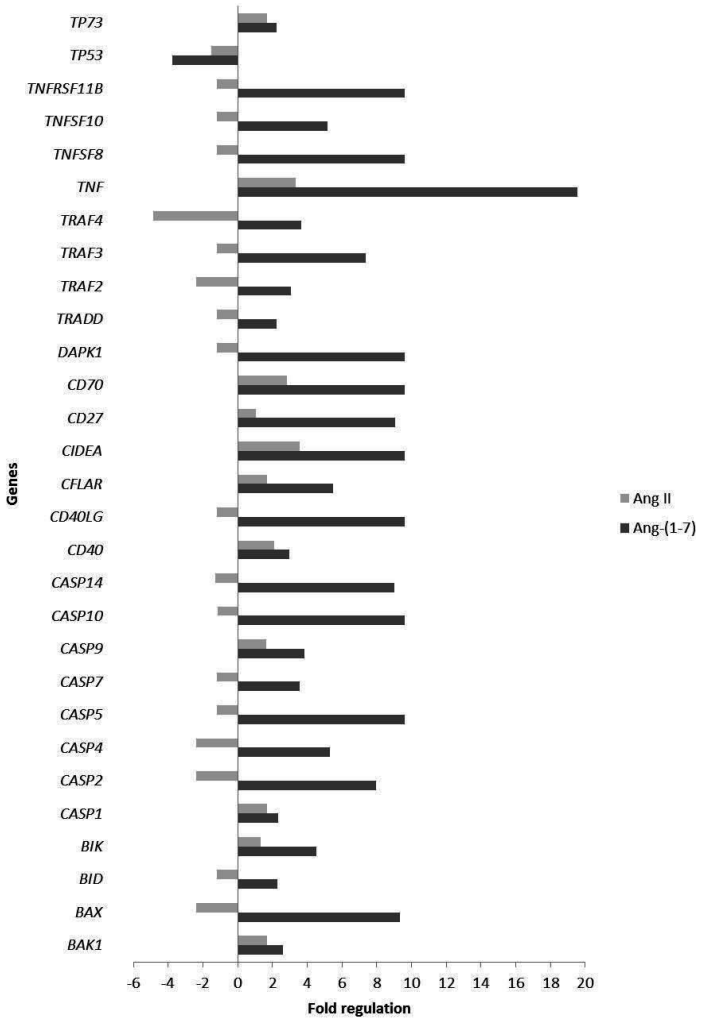

Figure 4. Pro-apoptotic genes with altered expression after Ang-(1-7) and Ang II treatment. T47D cells were treated for 48 hours with the peptides and gene expression was verified by qPCR. Fold-regulation values greater than 2 indicate up-regulation and fold-regulation values smaller than −2 indicate down-regulation.

Pro-apoptotic genes of the *BCL*-2 family, such as *BAX*, *BID*, *BIK* and *BAK*, were up-regulated after treatment with Ang-(1-7). The induction of apoptosis by these genes has been fully demonstrated in several studies [42]-[44].

Estrogen-dependent cancers, such as breast and endometrial cancer, exhibit high expression of the *BCL*-2 gene (anti-apoptotic) and possibly for this reason, have low level of apoptosis [45]-[47]. In this, the *BCL*-2 gene was overexpressed by Ang-(1-7), but the apoptotic percentage of T47D cells was significantly greater when compared to the control group. We infer if the increased expression of the *BAX* gene (pro-apoptotic), which was up-regulated after treatment with Ang-(1-7), might have antagonized the protective effect of *BCL*-2. This effect has been shown in some studies with the *BAK* gene (which functions similar to the *BAX*). The overexpression of this gene induces apoptosis in cancer cells even in the presence of high expression levels of anti-apoptotic genes of the Bcl-2 family [48] [49].

BIK has the capacity to induce apoptosis in mammary adenocarcinoma cells [50] and sensitize tumor cells to chemotherapeutic agents [51]. This corroborates with the findings of our study and opens the possibility of the use of Ang-(1-7) as a chemotherapy sensitizer.

The genes of the TNF superfamily, including *TNF*, *TNFSF*10, *TNFSF*8 and *TNFRSF*11*B*, encode apoptotic proteins [52] [53]. These genes were overexpressed by Ang-(1-7).

Ang-(1-7) induced the expression of *CD*40 and *CD*40*L* genes. Studies conducted in pancreatic cancer cell lines demonstrated that the expression of these genes have growth inhibitory effect [54]-[56].

CFLAR, *CIDEA*, *DAPK*1, *FASLG*, *CD*27 and *CD*70 genes induce cell death [57]-[60]. *TRADD*, *TRAF*2, *TRAF*3 and *TRAF*4 are genes that encode proteins that increase apoptosis rate [61] [62]. All these genes were up-regulated after treatment with Ang-(1-7) in the present study.

The TP53 gene, encoding the p53 protein, is mutated in line T47D and perhaps for this reason was not observed increased expression of this gene suppressor tumor after treatment with the Ang-(1-7). However, there was a significant increase in expression of TP73, encoding a protein of very similar role to TP53. The TP73 gene is capable of acting on *BAX*, *BCL2L*1, *MCL* and Caspase 9 [63], inducing apoptosis in tumor cells.

In this study, some genes considered anti-apoptotic or apoptosis suppressing, such as *AKT*1, *BAG*4, *BCL2A*1, *BCLAF*1, *BFAR*, *FADD*, *FAS* and *XIAP* [64]-[72] were down-regulated by Ang-(1-7).

The *AKT*1 gene encodes the Protein Kinase B, which is capable of inhibiting the protein BAD through phosphorylation and induce anti-apoptotic effects [73].

The *XIAP* gene protects endometrium carcinoma cells against various pro-apoptotic agents [74] [75]. The use of RNA interference (siRNA) against XIAP induces apoptosis and inhibits potential tumor growth in breast cancer cells [76].

In summary, apoptosis was increased and the expression of several genes related to apoptosis was changed after Ang-(1-7) treatment.

5. Conclusion

Our results support the hypothesis that Ang-(1-7) could change the expression of several apoptosis-related genes, interfering directly in the molecular pathways associated with the survival of breast cancer cells. *In vivo* assays are being conducted to confirm the potential of this hormone as an antitumor agent.

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List of Abbreviations Used

ACE: Angiotensin-Converting Enzyme;
Ang II: Angiotensin II;
Ang-(1-7): Angiotensin-(1-7);
cDNA: Complementary DNA;
DMEM: Dulbecco's Modified Eagle Medium;
FBS: Fetal Bovine Serum;
PCR: Polymerase Chain Reaction;
qPCR: Real-Time PCR or Quantitative PCR;
RNA: Ribonucleic Acid;
RAS: Renin-Angiotensin System.

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