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Regulation of TSH Receptor Autoantibodies by a long Non-Coding RNA (*Heg*) and Cdk1- A Review

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Authors' contributions

The original work was carried out in collaboration between all authors. Author NJC wrote the first draft of the review. All authors read and approved the final manuscript.

Review Article

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ABSTRACT

Aims: A substantial part of the genome is transcribed in non-coding RNAs. We review our finding of a long non-coding RNA (designated *Heg*) in mononuclear cells (MNC) and regulation of TSH receptor autoantibodies (TRAb).
Results: The *Heg* RNA transcript in MNC is negatively correlated with TRAb in patients with early and untreated Graves' disease. In treated patients and in controls *Heg* correlated negatively with *CD14* mRNA. Transfection studies with fragments of *Heg* added to MNC (exogenous *Heg*) decreased *CD14* mRNA in MNC and increased gene expression of *RIG-I*, *TLR7 and IFN-γ*. *Heg* is likely to activate TLR7 receptors. CD14 is a co-receptor of TLR7. Decrease in gene expression of *CD14* after *Heg* is a sign of differentiation of MNC to dendritic cells. This may reduce surface expression of CD14, cytokine responses and the responsiveness to TSH receptor antigens. Thus the relationship between TRAb and lnc *Heg* RNA is most likely explained by receptor cross-interference. *Cdk1* mRNA (an index of cell cycle activity) is positively related with TRAb. *Cdk1* mRNA and TRAb but not *Heg* decreased significantly during antithyroid treatment. *Cdk1* decreased to values below normal.
Conclusion: Thus both *Heg* RNA and *Cdk1* may regulate the level of TRAb but by two different mechanisms.

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Keywords: Antithyroid drugs; autoimmunity; CD14; Cdk1; receptor cross-interference; long non-coding Heg RNA; TSH receptor autoantibodies.

ABBREVIATIONS

RIG-I: Retinoic-acid-inducible gene 1; IFIT: Interferon-induced protein with tetratricopeptide repeats; IFN: interferon; α,β,γ; Amol: Attomol; Zmol: Zeptomol.

1. INTRODUCTION

A substantial part of the genome is transcribed in non-coding RNAs. Many studies have focused on miRNAs, which are important for cell proliferation and cancer. Recently two studies of miRNA profiles have been reported in thyroid diseases [1-2]. We review our finding of a lnc (long non-coding) RNA (designated *Heg*) in peripheral blood mononuclear cells (MNC) and regulation of TSH receptor autoantibodies (TRAb). We have not been able to find information about other lnc RNAs related to the physiology or pathophysiology of TRAb, but the function and regulatory principles of lnc RNAs have recently been summarized [3-6]. Some lnc RNAs are rapidly degraded and important for activation of inducible genes. Other lnc RNAs are very stable [7-8]).

In our laboratory quantification of RNA was performed by RT-PCR-HPLC [9-10]. HPLC was applied to separate the peak value of the specific standard and the RNA to be measured. All chromatograms were examined graphically on a computer screen. During a study of *Foxp3 mRNA* in MNC we observed on the chromatogram a RNA fragment without annotation. The area of the peak correlated with gene expression of *CD14 mRNA* as measured in a small group of subjects. The sequence was localized by a BLAST search to a clone from the HUGO project on chromosome 1 and designated *Heg* (4002 bases; GenBank EU137727). *Heg* RNA is a single stranded RNA fragment and antisense to and overlapping a major part of exon 7 of the *Nucks* mRNA (GenBank NM_022731.4). Nucks, nuclear ubiquitous casein kinase substrate, is known to play a major role in transcription regulation and is a substrate for Cdk1 [11]. *Heg* is considered to be a lnc RNA, and we have not been able to transcribe *Heg* by oligo (dT) priming. *Heg* includes an open reading frame (ORF) of 97 amino acids. A lnc RNA may contain such an ORF by chance and many well-characterized lnc RNAs do indeed contain relatively long ORFs. A protein corresponding to the 97 amino acids has not been isolated. Furthermore the relationship between *Heg* RNA and *CD14* mRNA may be imitated by fragments of *Heg* RNA.

To examine the possible role of *Heg* in the development of autoimmunity we studied TRAb in patients with Graves' disease [12-14]. Our studies included 17 patients with early, untreated Graves' disease, 20 patients who had been treated with antithyroid drugs for several months and 18 normal subjects. Additional samples were obtained from normal subjects for incubation studies. We also analyzed different types of non-activated MNC. Information about subjects included in the study and a description of methods applied for quantification of RNA have been presented earlier [9,15].

2. TRAb AND RELATIONSHIP WITH LONG NON-CODING *HEG* RNA AND *CD14* mRNA

In the first part of the study we examined, if TRAb was correlated with lnc *Heg* RNA. There was a negative and significant relationship in patients with untreated Graves' disease

between TRAb and lnc *Heg* RNA amol/µg DNA [9]. We have not found any other factor, which correlated with TRAb in untreated patients except *Cdk1 mRNA*. *Cdk1 is* an index of cell cycle activity [16] and will be discussed later. Including *Cdk1* mRNA zmol/µg DNA in the regression analysis increased the r value (numerically) from -0.61 to -0.83. Fig. 1 shows the negative relationship observed between log TRAb and the ratio *Heg* RNA/Log *Cdk1* mRNA [15]. There was no significant relationship between TRAb and *Heg* RNA in treated patients with Graves' disease. A negative relationship was observed, however, between *CD14* mRNA and lnc *Heg* RNA. Cd14 is a co-receptor of toll-like receptor 4 (TLR4). It has recently been reported, that CD14 is also a co-receptor of TLR7 and is required for TLR7 dependent cytokine responses [17-18]. Results were approximately similar in treated patients and in controls. In the combined group of subjects we observed a strong negative correlation between *Cd14* mRNA and lnc *Heg* RNA. We also included *Nucks* mRNA in the analysis, to see if lnc *Heg* was dependent on the transcription rate of Nucks. *Nucks* mRNA was positively related to *Heg* RNA but not to *CD14*. The best description of the relationship between *CD14* mRNA and *Heg* RNA was obtained, if a correction was made for the influence of *Nucks* on the *Heg* level. Thus subjects with a high lnc *Heg* RNA/*Nucks* mRNA ratio had low levels of *CD14* mRNA [9].

3. TRANSFECTIONS STUDIES

These relationships do not necessarily imply any causal relationship. We did therefore a number of experiments, where MNC were incubated with a single-stranded fragment of *Heg* RNA. One of these experiments is shown in Fig. 2 [9]. No significant change was observed in the control experiment, but in the experiment with *Heg* RNA *CD14* mRNA decreased from 23±1.2 to 1±0 amol/µg DNA at 24 hours. Exogenous RNA is not taken up by MNC unless a transfection agent is added. No effect of fragments of Heg was observed unless a transfection agent was added to the incubation medium. We applied lipofectamine, which alters the cellular plasma membrane allowing nucleic acids to cross into the cytoplasma. Lipofectamine had no effect on basal levels of *Heg* demonstrating that it was of endogenous origin. Transfection studies with fragments of *Heg* and lipofectamine added to MNC clearly increased *Heg* RNA in MNC and decreased *CD14* mRNA. A similar decrease in *CD14* mRNA was also observed after incubation with antisense *Heg* RNA derived from the *Nucks* sequence demonstrating that the response was not dependent on the specificity of the sequence.

TSH receptor autoantibodies and gene expression of *Heg* and *Cdk1*

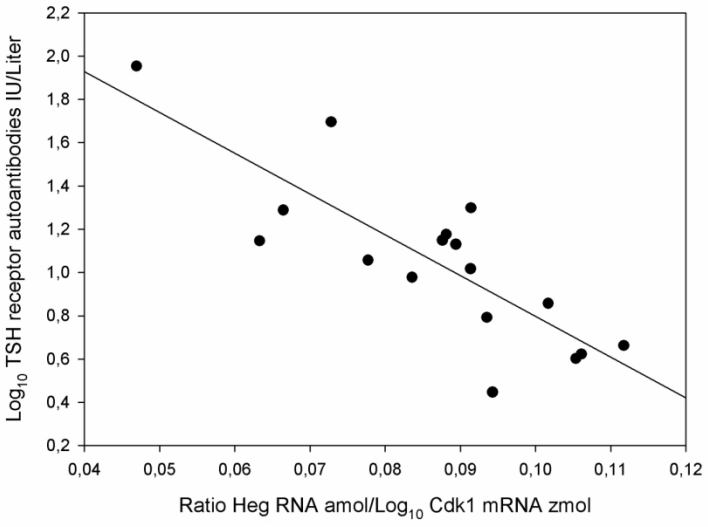

Fig. 1. The relationship between log_{10} TSH receptor autoantibodies (TRAb) IU/Liter and the ratio *Heg* RNA amol per µg DNA/Log_{10} *Cdk1* mRNA zmol per µg DNA. R=-0.82; (*P*<.001). Patients with early and untreated Graves' disease. (Reproduced with permission from Christensen et al. [15])

Effects of *Heg* RNA on gene expression of *CD14*

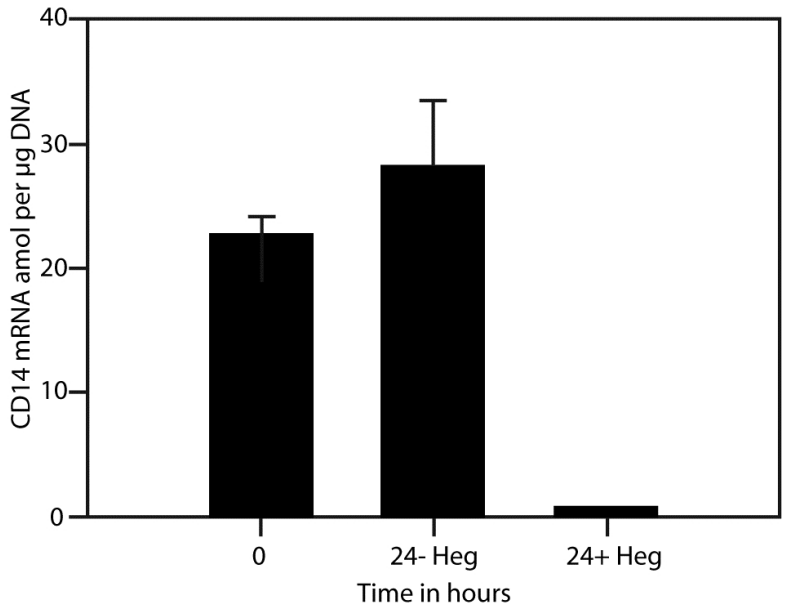

Fig. 2. *CD14* mRNA amol per µg DNA plotted on the ordinate. Results are basal values and values after 24 h incubation without (-) and with (+) addition of a *Heg* fragment (*P*<.001). (Reproduced with permission from Christensen et al. [9])

4. TOLL-LIKE RECEPTOR 7

The TLR7 protein is a member of the Toll-like receptor family. It recognizes single stranded RNA in the endosome (viral genomes) and activates cytokines. It is important for innate immunity. RIG-I, IFIT and IFN proteins are all important for activity against viral infections. Rig-I is a cytoplasmic receptor and function in the same way as TLR7 as a sensor for recognition of viral RNA. Both RIG-I and TLR7 activate gene expression of IFN. *Heg* RNA in patients with untreated Graves' disease showed a weak but significant positive correlation with gene expression of *TLR7* and *RIG-I* ($P<.01$ and .02, respectively). *Heg* RNA fragments increased gene expression of both *TLR7* and *RIG-I* approximately five fold. Both endogenous *Heg* (coming from inside the cell) and exogenous *Heg* (added to cells with lipofectamine) were probably detected by RIG-I and other factors like IFIT and activated TLR7 in the endolysosome (see below). TLR8 is also considered to be active against single stranded RNA, but *TLR8* mRNA did not increase. We also measured *TLR7* mRNA in different non-activated MNC types obtained from healthy subjects. CD14+ cells had high levels of *TLR7* mRNA as compared with other cell types (CD14+cells 2838±34; dendritic cells 346±14; CD8 cells 0±0 expressed as the peak area/μg RNA). Decrease in *CD14* mRNA after exogenous *Heg* (meaning *Heg* coming from outside the cell in transfection studies with lipofectamine added) is a sign of differentiation of blood monocytes to dendritic cells.

IFN-α mRNA increased significantly in response to exogenous *Heg*, and correlation was observed between *IFN-α* mRNA and the corresponding IFN-α protein. *IFN-α* mRNA was not detectable in the basal state. *IFN-γ* mRNA increased 22 and 137 fold 6 and 24 hours after addition of exogenous *Heg*. There was also a positive relationship in normal subjects between endogenous levels of *IFN-γ* mRNA amol/μg DNA and endogenous *Heg*. *IFN-γ* mRNA values ranged from 0.02 to 0.18 amol/μg DNA.

5. MECHANISMS

What is the explanation of the negative relationship observed between TRAb and lnc *Heg* RNA? Lnc *Heg* RNA is negatively correlated with TRAb or *CD14* mRNA in untreated patients with Graves' disease and in treated patients and controls, respectively. Transfection studies with fragments of *Heg* also decreased *CD14* mRNA and increased gene expression of *TLR7, RIG-I* and *IFN-γ*.

Recent studies have shown that some lnc RNAs are stable, but half-lives may vary [8]. Some lnc RNAs rapidly broken down in the nucleus may represent noise. Other lnc RNAs are expressed proximal to inducible genes and may regulate the chromatin state. The clearance of these genes by decapping results in gene activation [7]. Exogenous *Heg* (+ lipofectamine) added to MNC was probably fused with the cell membrane and transported to the endosome, where it activated TLR7. The marked increase in *RIG-I* mRNA suggests that exogenous *Heg* was also detected in the cytoplasma [19-20]. It is not clear at present how endogenous *Heg* (coming from inside the cell), activated RIG-I and TLR7, because there are several RNA degrading pathways in the cytoplasma [21-22]. The response pattern to endogenous and exogenous *Heg* was, however, rather similar and both RNAs were associated with a decrease in *CD14* mRNA. The effect of *Heg* was not dependent on its specific sequence but more on its molecular pattern as a single stranded RNA molecule. This is also so with viral RNA. Pattern recognition receptors sense molecular signatures associated with viral RNA.

It has recently been reported that CD14 is a co-receptor not only of TLR4 but also of TLR7 and is required for TLR7 dependent cytokine responses [17-18]. Control of *TLR7* expression is important to restrict autoimmunity and dendritic cell expansion [23]. The negative relationship between *Heg* and *CD14* mRNA and the decrease in *CD14* mRNA after transfection with *Heg* RNA is likely to be a sign of differentiation of monocytes to dendritic cells. Upon differentiation the cell surface expression of CD14 is lost, whilst CD209 expression is increased [24]. There is likely to be a continuously small production of *Heg* RNA in MNC, which decreases gene expression of *CD14* mRNA, reduces cytokine secretion and cytokine responses and this may reduce responsiveness to TSH receptor antigens. Our findings are most likely explained by receptor cross-interference. Small increments in the flow of lnc *Heg* RNA to the endolysosome may also reduce autoantibody production for instance in early juvenile diabetes. Receptor cross-interference has recently been reported by Negishi et al. [25]. These authors showed that recognition of double-stranded RNA by RIG-I-like receptors suppresses TLR induced expression of interleukins 12 and 23 and antibacterial responses.

6. *CDK1* mRNA AND ANTITHYROID TREATMENT

It is well known that TRAb decreases during treatment with antithyroid drugs. As mentioned previously 20 patients were studied after treatment had been initiated. *Heg* RNA concentrations in MNC were not measured before treatment, but their TRAb levels were available. Expectedly TRAb had decreased approximately 50% (from a median level of 13.5 to 6.5 IU/l; $P < .004$). This decrease in TRAb during treatment cannot be explained by *Heg*, which remained unchanged.

We have previously shown that *Cdk1* was positively related to TRAb (see above) and we wanted to see if gene expression of *Cdk1* changed during treatment. Cdk1 is a cyclin-dependent kinase, which is necessary to drive cell division. Furthermore, the Nucks protein plays a major role in transcription regulation and is a substrate for Cdk1. Concentrations of *Cdk1* mRNA were significantly reduced in the group of treated patients to 43% as compared with untreated patients and normal subjects (Table 1; ANOVA ($P < .001$) [15]. Calculated TRAb values obtained from the regression line (relating TRAb to *Heg* RNA and *Cdk1* mRNA) after an assumed reduction in *Cdk1* mRNA values of 50% also resulted in a decrease in TRAb of 50%. Note that *Cdk1* mRNA decreased to levels significantly below levels observed in normal subjects. Concentrations of *Cdk1* mRNA were not significantly different in untreated patients and in normal subjects. This suggests that the decrease in TRAb during treatment with antithyroid drugs may be due to a reduction in cell cycle activity. The decrease in *Cdk1* during antithyroid treatment was in all probability a pharmacological effect of antithyroid treatment. Clearly further studies may be of interest especially in vitro studies to examine, if addition of antithyroid drugs to MNC in vitro decreases *Cdk1* mRNA. It is unclear at present, if the effect of antithyroid drugs on *Cdk1* mRNA is specific for Graves' disease.

Table 1. *Cdk1* mRNA concentrations expressed in zmol/µg DNA (median and 25% and 75% ranges) in untreated and treated patients with Graves' disease and in controls. (reproduced with permission from Christensen et al. [15])

Untreated patients	Treated patients	Normal subjects
33 (22 to 39)	13 (10-17)*	27 (18-34)

**Significantly different from the two other groups (ANOVA; P < .001).*

7. LONG NON-CODING *HEG* RNA AND SUSCEPTIBILITY GENES

A number of genes may contribute to the development of Graves' disease 1) genes from the *HLA-DR* gene locus 2) immune-regulatory genes (*CD40, CTLA-4 and PTPN22*) and 3) thyroid specific genes. Autoantigens may bind to receptors on T-cells, which have escaped tolerance [12-14]. CTLA-4 and PTPN22 genes are both negative regulators of T-cell activation and CD40 is important for activating of B-cells. Polymorphism of these genes may influence TRAb production. Genetic variations in TLR receptors may also contribute to disease [26]. There is no evidence that lnc *Heg* RNA has any specific effect on the development of Graves' disease. Lnc *Heg* RNA is related to the Nucks gene, but it is not the *Nucks* mRNA. Lnc *Heg* RNA may perhaps influence the early inflammatory response during the development of Graves' disease. Cdk1 is proinflammatory and may activate B-cells. Lnc *Heg* RNA is likely together with gene expression of *Cdk1* and other factors to regulate the level of TRAb and to some extent disease activity. Relationships between lnc *Heg* and the above mentioned susceptibility genes deserve further investigations. Our results suggest that decrease in TRAb during treatment with antithyroid drugs may be due to a decrease in *Cdk1 mRNA* to levels below normal. Clearly further studies are necessary to confirm this hypothesis.

8. CONCLUSIONS

The present study indicates that two different factors, a lnc *Heg* RNA and *Cdk1* mRNA may regulate TRAb. *Heg* may activate TLR7 in the endolysosome and decrease gene expression of *CD14* mRNA. It is likely to be a sign of differentiation of monocytes to dendritic cells. This change may reduce the surface expression of CD14, decrease cytokine secretion and the responsiveness to TSH receptor antigens. Decrease in TRAb during treatment with antithyroid drugs cannot be explained by *Heg*. *Cdk1* mRNA, which is an index of cell cycle activity, decreased significantly during treatment to values below normal. Gene expression of lnc *Heg* RNA and *Cdk1* mRNA may both regulate the level of TSH receptor autoantibodies but by two different mechanisms. The correlations observed about the decrease in TRAb were not a direct immunologic mechanism to regulate the particular autoantibody production, but an indirect cellular mechanism(s) resulted in the autoantibody decrease. Therefore it may be of interest to study the same mechanisms for example in subjects at risk of developing Type 1 diabetes.

CONSENT

Written Informed consent was obtained from all subjects, who participated in the original studies. The study protocols were approved by the Ethics Committee of Copenhagen County.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Ethics Committee of Copenhagen County and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

None. No competing financial interests exist.

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