CORRESPONDENCE

The "*NF-κB interacting long noncoding RNA*" (*NKILA*) transcript is antisense to cancer-associated gene *PMEPA1* [v1; ref status: indexed, http://f1000r.es/5aq]

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Abstract

This correspondence concerns a recent publication in *Cancer Cell* by Liu et al.[1] who analyzed a long noncoding RNA (lncRNA) that they designated "*NKILA*". Liu et al. found that *NKILA* (1) is upregulated by immunostimulants, (2) has a promoter with an NF-κB binding motif, (3) can bind to the p65 protein of the NF-κB transcription factor and then interfere with phosphorylation of IκBα, and (4) negatively affects functions that involve NF-κB pathways. And, importantly, they found that (5) low *NKILA* expression in breast cancers is associated with poor patient prognosis. However, they entirely failed to mention *PMEPA1*, a gene which runs antisense to *NKILA*, and the expression of which is associated with several tumors and which encodes a protein that participates in immune pathways.

The *PMEPA1* locus, including its promoter region, which Liu et al.[1] only discuss in regard to *NKILA*, is highly conserved through evolution. Our impression is that *NKILA* emerged only later in evolution, possibly as an additional means of *PMEPA1* regulation. Liu et al., however, only consider direct binding between *NKILA* and NF-κB as the mechanism for their *in vivo* observations of *NKILA* function, but do not provide solid evidence for their model. If *in vivo* observations by Liu et al. could be explained by *NKILA* regulation of *PMEPA1*, it would contribute to the establishment of *PMEPA1* as an important topic of cancer research. We feel that the herein presented discussion is necessary for a correct interpretation of the Liu et al. article.

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version 1 published 22 Apr 2015	✓ report	✓ report

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Comments (0)

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Correspondence

Liu *et al.*[1] investigated breast cancer cell lines for possible association of known long noncoding transcripts with immunostimulation. They found that an unspliced lncRNA, which they designated "*NKILA*" (represented by large yellow box in Figure 1), could be upregulated by several immune agents. However, they did not mention the existence of a reported spliced form of *NKILA* (GenBank accession DA866558, see Figure 1), or, - our major criticism - that *NKILA* is divergently transcribed from *prostate transmembrane protein, androgen induced 1* (*PMEPA1*) and antisense to some of its transcripts (see Figure 1). One of the alternative names for *PMEPA1* is *solid tumor associated gene 1* (*STAG1*)[2], and its expression was found upregulated in several tumors including breast cancer (e.g., references 2 and 3). Liu *et al.*[1] found that the *NKILA* promoter region contains an NF-κB binding motif (Figure 1), which according to our analysis is rather well conserved among eutherian mammals (Supplementary file S1). However, whereas the *PMEPA1-001* transcript open reading frame and a part of the intergenic promoter region are highly conserved through evolution, this seems to be true to a lesser degree for *NKILA* equivalent transcripts (Supplementary file S1 and discussion therein). So, from the standpoint of evolution, a logical hypothesis for the function of the seemingly younger *NKILA* is its possible interference with *PMEPA1* expression[4,5].

PMEPA1 expression is strongly enhanced by TGF-β[6,7], something which agrees with the two SMAD binding element motifs that we found conserved in its promoter (Supplementary file S1). *PMEPA1* function is not well understood, but it is believed to encode a transmembrane protein that with its cytoplasmic domain can bind SMAD proteins and can positively affect activation of Akt[7]. Signaling pathways involving Akt and NF-κB are known to converge[8], which might be relevant for a possible indirect effect of *NKILA* through *PMEPA1* on NF-κB functions. PMEPA1 levels were reported to be high in invasive MDA-MB-231 breast cancer cells, and low in non-invasive MCF-7 and T47D breast cancer cells, agreeing with observations for aggressive versus non-aggressive tumors[9]. This is exactly opposite to the expression pattern observed by Liu *et al.* for *NKILA* in these cell lines and among tumors. *PMEPA1* knockdown has been found

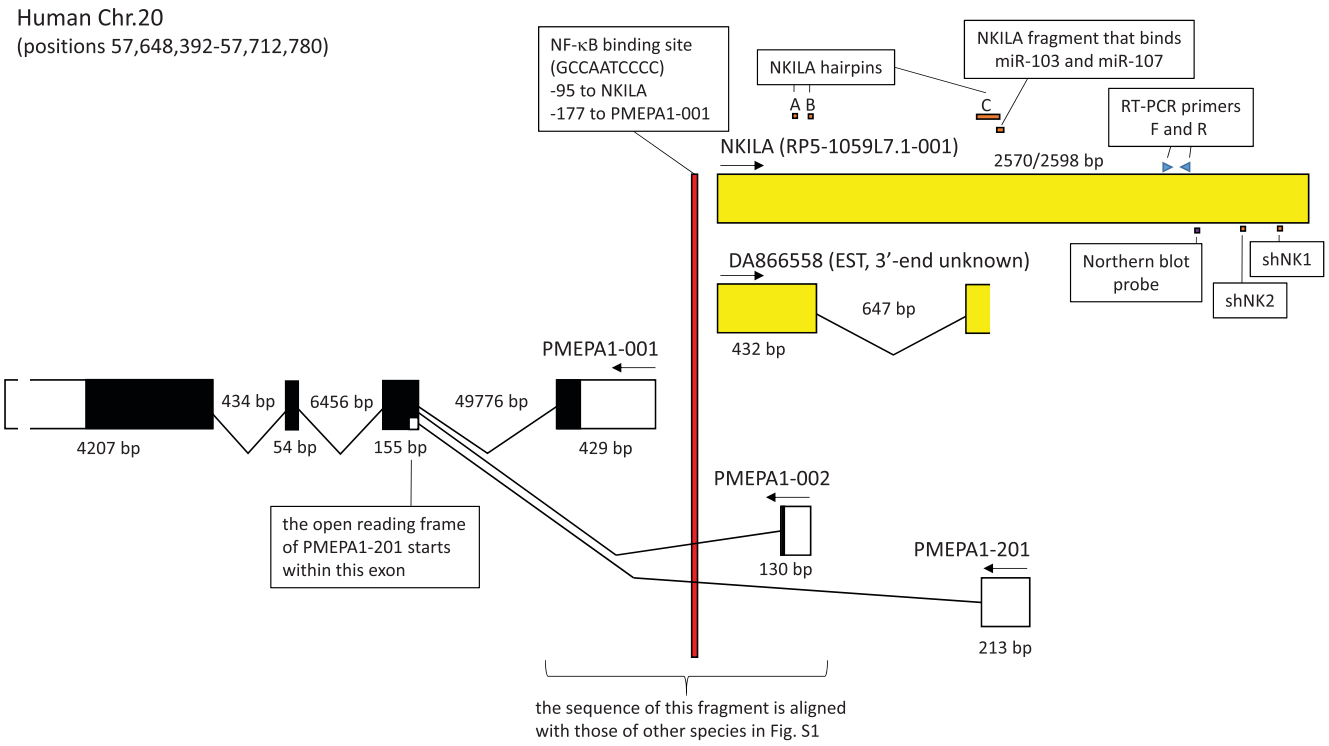

Figure 1. Schematic view of the *PMEPA1* plus *NKILA* region of human Chr. 20. The figure summarizes several data from the study by Liu *et al.* for *NKILA* and its promoter, while also showing overlapping transcripts that were neglected in that study. The *NKILA* transcript identified by Liu *et al.* roughly corresponds with transcript RP5-1059L7.1-001 as summarized in the GRCh38.p2 dataset of the Ensembl database (http://www.ensembl.org/index.html). GenBank accession DA866558 (RP5-1059L7.1-002 in Ensembl) contains an expressed sequence tag (EST) which represents the 5' end of a spliced transcript and for which the 3' end is not known. The depicted summary of the *PMEAP1* transcripts *-001*, *-002* and *-201*, is derived from the Ensembl database and agrees with GenBank reports; for additional variations of *PMEAP1* transcripts we refer to the Ensembl database. Exons are indicated by boxes, with protein coding regions in black. The 3' UTR of *PMEPA1* is not drawn in correct proportion to the other exon regions. Arrows indicate the direction of transcription, and genomic regions are measured in basepairs. The figure also shows from the Liu *et al.* report the positions of the NF-κB binding promoter element, the *NKILA* hairpin-prone regions, the *NKILA* region that binds miR-103 and -107, the *NKILA* binding sites for the Northern blot probe and RT-PCR primers, and the shNK1 and shNK2 regions from which sequences were derived for cloning into shRNA constructs.

to be able to attenuate growth and motility of MDA-MB-231 breast cancer cells[9], which is interesting since Liu *et al.*[1] found that forced *NKILA* expression (which might knockdown *PMEPA1* expression) in MDA-MK-231 cells achieves similar effects. Consistent with these findings is the observation that high *PMEPA1* expression in breast cancer is associated with poor patient prognosis[7], and high *NKILA* expression with better patient prognosis[1]. Although there are also published *PMEPA1* reports which are harder to reconcile with such a model (e.g. reference 10), and which are hard for us to validate, at least the above selected set of data suggests that *NKILA* has a negative effect on *PMEPA1* function. More research on both *NKILA* and *PMEPA1* will be necessary before conclusions can be made, but for now the possibility that *NKILA* can downregulate *PMEPA1* expression appears to be a reasonable model[4,5].

Liu *et al.*[1] concluded that *NKILA* interferes with pathways that involve NF-κB function, and we feel that in essence this conclusion can be believably deduced from their abundant experimental data. However, mechanistically Liu *et al.* only consider a direct interaction with NF-κB components and fail to consider an indirect effect through *PMEPA1* regulation. Liu *et al.*[1] did find direct binding between the NF-κB component p65 and *NKILA*, but whether this can be considered as evidence of physiological specificity of *NKILA* for NF-κB is questionable. When they analyzed proteins that they could pull down with *NKILA* they only compared different NF-κB pathway components using Western blot analysis[1]. In addition, when they quantified *NKILA* by RT-PCR on genetic material that co-precipitated with NF-κB factors, they did not exclude the possibility that they might be measuring genomic *NKILA* DNA[1].

Liu *et al.*[1] mapped the *NKILA* interaction with p65 to hairpin-prone regions A and B, and showed that the hairpin-prone region C can interact with NF-κB pathway factor IκBα (for hairpin-prone region locations see Figure 1). By mutation analysis, Liu *et al.*[1] found that all these three hairpin-prone regions are important for the inhibitory effect of *NKILA* on NF-κB activity. Although the relevance of this overlap is not clear, we point out that all three identified hairpin-prone regions, and also the region which Liu *et al.*[1] found to confer sensitivity to miRNA induced degradation, overlap with known *PMEPA1* transcript regions (Figure 1).

As an additional remark, we would like to state that the somewhat discussable manner in the way Liu *et al.*[1] performed or described some of their experiments (see our comments in Supplementary file S2) does not help to convey the image of a study which is solid in its quantitative aspects. However, despite our criticism, it is only fair to state here that according to our judgement the very elaborate study by Liu *et al.*[1] believably shows (1) how *NKILA* can bind (*in vitro*) to NF-κB, (2) that *NKILA* can interfere with functions that involve NF-κB pathways, and (3) that low *NKILA* expression predicts poor clinical outcome in patients with breast cancer. But they should mend the open ends, which means providing more evidence of the specificity of the *NKILA* binding to NF-κB, and to take the possible effects of *NKILA* through regulation of *PMEPA1* into consideration. In regard to the more general claim by Liu *et al.*[1] that there exists "a class of lncRNAs that regulate signal transduction at post-translational level", we believe as before[11] that such a conclusion needs more evidence than currently has been presented. We hope that our present discussion leads to an increased interest in the *PMEPA1-NKILA* locus, because whatever mechanism may be correct, Liu *et al.* did provide evidence that this locus is clinically important in breast cancer.

Author contributions

JMD initiated the study. DBA and JMD performed the analysis. JMD wrote the article with help of DBA. Both authors critically edited the correspondence and agreed to the final content.

Competing interests

No competing interests were disclosed.

Grant information

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Supplementary material

Supplementary file S1.

Alignment of *PMEPA1-NKILA* promoter region sequences of representative animals and deduced PMEPA1 amino acid sequences for the species compared.

Click here to access the data. http://dx.doi.org/10.5256/f1000research.6400.s45984

Supplementary file S2.

List of issues regarding the *Liu et al. (2015). Cancer Cell 27, 370–381* paper which in our opinion need attention.

Click here to access the data.

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Our laboratory (Saikumar) published two manuscripts on the PMEPA1/TMEPAI gene product that acts as a molecular switch in converting transforming growth factor-beta (TGF-β) from a tumor suppressor to a tumor promoter by suppressing canonical Smad signaling and promoting non-canonical PI3K/Akt signaling using breast cancer models. We have been invited to review and comment here on the correspondence paper by Dijkstra and Alexander. The correspondence titled "The NF-kB interacting long noncoding RNA (NKILA) transcript is antisense to cancer-associated gene PMEPA1" by Dijkstra and Alexander, provided an alternate explanation to the results presented by Liu *et al* in a recent publication "A cytoplasmic NF-kB interacting long noncoding RNA blocks IkB phosphorylation and suppresses the breast cancer metastasis" in Cancer Cell (27: 370-381, 2015). Although the novel finding of the later is that the long noncoding RNA designated NKILA is upregulated by immunostimulants due to the presence of a NF-kB binding motif in the promoter region of this gene which is located in the human chromosome 20q13.31. NKILA driven by NFkB acts as a negative feedback regulator of NFkB activation through a direct interaction with NF-kB p65-IkB complex, which was the basis for the negative effects on the events mediated by NF-kB signaling pathways. Importantly, these authors either ignored or failed to notice the similarities between NKILA and PMEPA1, another gene present in the same locus of chromosome 20. A simple BLAST search would have indicated the similarities between these two genes and their locations. Interestingly, NKILA appears to fit the description of a head-to-head antisense RNA for PMEPA1.

Dijkstra and Alexander rightly noticed this omission and highlighted in their correspondence the following: i) Both NKILA and PMEPA1 genes are in anti-sense and sense orientation in the same region in chromosome 20; ii) Based on the overlap, Dijkstra and Alexander proposed a provocative hypothesis that NKILA transcript may have the potential to regulate PMEPA1. The authors are correct in pointing out that Liu *et al.* completely overlooked the existence of PMEPA1 and did not take into account in their discussion of the mechanism involving NKILA; iii) They rightly state that indeed future experiments should verify a link between NKILA and PMEPA1. Some of the issues they raised in the supplementary file 2 were scientifically valid. This correspondence is purely theoretical and short of much needed experimental data to show that NKILA negatively regulates PMEPA1 mRNA levels. However, in defense of the authors of the original paper, there is no evidence to suggest that immuno-stimulants like TNF-α and LPS can induce PMEPA1.

Overall the correspondence merits indexing with high importance because it will initiate research activity to identify the link between these two genes during inflammation.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 27 April 2015

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Peter G. Zaphiropoulos
Department of Biosciences and Nutrition, Karolinska Institute, Huddinge, Sweden

The Dijkstra and Alexander correspondence suggests that the noncoding NKILA RNA effects on NF-κB signaling in breast cancer cells of the recent Liu *et al.* Cancer Cell publication may be partly mediated via the protein coding PMEPA1 gene, which is positioned in an antisense orientation (head to head) to the NKILA locus.

While the authors do not question the main thesis of the Liu paper, namely the direct interaction of the NKILA RNA to p65 that leads to inhibition of IκB phosphorylation, they highlight certain published data, which are indicative of a role of PMEDA1 in breast cancer and, interestingly, of a possible reciprocal regulation of PMEDA1 and NKILA.

In my opinion, the hypothesis put forward by Dijkstra and Alexander is of interest and should be experimentally addressed to test whether there is indeed an interplay between the sense-antisense PMEDA1-NKILA gene pair, which may further expand the biological roles and mechanisms of action of the NKILA noncoding RNA. In particular, I would like to see the impact of PMEDA1 depletion in the observable NKILA biological effects and vice versa the impact of NKILA depletion in PMADA1 gene expression.

Clearly, a state of validating or refuting a hypothesis by experimental means is in front of the scientific community.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response 27 Apr 2015

Johannes M. Dijkstra, Fujita Health University, Japan

Dear Dr. Zaphiropoulos,

Thank you for your review and your approval. Since you are an expert in RNA functions, we are very happy that you consider our hypothesis of *NKILA* possibly affecting PMEPA1 function to be a realistic possibility.

However, you are incorrect in that we do not question the main thesis of the Liu et al. paper. We agree that for the direct *NKILA* to NF-kB binding mechanism, Liu et al. provided a lot of support by

in vitro experiments. However, in our opinion, they did not provide the necessary conclusive *in vivo* experimental evidence for making a new model on lncRNA function. So we agree that they proved that *NKILA* can bind to NF-kB, but we say that they did not provide evidence that it does so in a specific or functional manner *in vivo*. Their paper is difficult to interpret because of the many experiments they did, combined with rather frequent incomplete descriptions of materials and methods (some of those issues we listed in our supplementary file S2). However, despite some uncertainties about what they actually did, a very critical flaw running through a large part of their paper appears to be the inability to distinguish cDNA from genomic DNA by their RT-PCR method. We would be interested to hear from you, as an RNA expert, whether you believe that our criticism of the Liu et al. article is correct.

We are sorry that you misinterpreted our story. If the other reviewers will have a similar misinterpretation, we will have to write our criticism of the Liu et al. paper in a more explicit way.

Sincerely,

also on behalf of David B. Alexander,

Johannes M. Dijkstra

Competing Interests: No competing interests were disclosed.

Referee Response 29 Apr 2015

Peter G. Zaphiropoulos, Karolinska Institute, Sweden

Dear Johannes (and David),

Thank you for your post.

In my reviewing of your correspondence I tried to distil its essence in terms of biological significance. In this spirit of constructive refereeing, the take-home message, in my opinion, is to test the possible interplay of the antisense protein-coding gene and the NKILA noncoding RNA, and urge you to engage in such experimental approaches to push science forward.

Concerning, your criticism of technical aspects of the Liu et al paper, I am not so sure. For example, your claim in supplementary file S2 and in your post that “RT-PCR amplification of an intronless sequence as done by the authors (see Figure 1) should consider the possibility of DNA contamination” is not in-line with the data of the Northern analysis depicted in the same Figure, panel A, which are consistent with the qRT-PCR data of Figure 1, panel C. Thus, my conclusion is that their qRT-PCR assay is robust enough and detects cDNA, as apparently their RNA preparations are essentially free of genomic DNA contamination.

Finally, I believe that it is appropriate to contact the authors of the Liu et al paper, either directly or via Cancer Cell, to express any additional concerns that you may have.

Best wishes,

Peter

Competing Interests: No competing interests were disclosed.

Author Response 29 Apr 2015

Johannes M. Dijkstra, Fujita Health University, Japan

Dear Peter,

Thank you for your answer. But I like to disagree with your technical assessment of "essentially free of DNA contamination", since the methods they say to have used do not provide such material. For which of the assays, if for any, the DNA contamination had a substantial impact on the results can't be known of course, but the authors should have excluded such possibility. To my knowledge, many scientists facing similar issues are very serious to get rid of the DNA, and it is at least peculiar that in the Liu et al. study such was not tried. Awareness of the problem would also have urged them to explain the sequences of the primers for ACTB gene amplfication, since now the ACTB versus NKILA comparison could theoretically be a measure for the amount of isolated DNA. I interpret your statement of "I am not so sure" as a correct assessment of the Liu et al. paper, which in its wordings does not seem to express the normal level of scientific insecurity itself.

Personally we are currently not so interested in doing lncRNA experiments ourselves, but we will start study of PMEPA1 at the protein level. At least that may be something good that has come of it.

Best wishes,

also on behalf of David,

Johannes

Competing Interests: No competing interests were disclosed.