

## Palindromati

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### ABSTRACT

This article describes a family of artificial heterotranscripts (RNA chimaeras) composed by thousands of *Genbank* sequences containing fragments or the complete *EcoRI*-like adapter acting as the palindrome linker *ctcgtgccgaattcggcaccgag*, binding together two or more genes that may be produced by different chromosomes. This happens due to current methodologies producing the reported sequences, found in the *Genbank*, in *Affymetrix* microarrays, and in many published articles reporting or using those sequences that include the *EcoRI*-like linker inside coding regions, and/or 5'UTR or 3'UTRs mRNA sites. This *EcoRI*-like linker and its heterotranscripts are here deemed as experimental artifacts, characterization that can be helpful to prevent errors, both in the studies of molecular mechanisms and in the drug discovery process.

**Key words:** *EcoRI*, Palindrome, Perilipin, *Genbank*, *Affymetrix*, microarray, species-specific.

### INTRODUCTION

It is vital in the discovery of new medical treatments to target precise molecules without having side effects for organic tissues. To accomplish this objective it is necessary a stringent quality control within molecular databases. This article describes the finding of numerous methodological artifacts reported to the *Genbank*. It is recommended a most carefully analysis of nucleic acid sequences for biological, medical and drug discovery purposes.

A single RNA binding in one-strand two different genes from two different human chromosomes (1) was the theoretical beginning for the study on heterotranscripts.

Here, I define *heterotranscripts* as chimaeras, sequences composed by fragments corresponding to two or more genes from the same or from different chromosomes.

I thought that such a phenomenon reported in reference (1) must have been reflected in a rational and logical combination of Intelligently Designed gene products (2, 3). As most of the vital molecules and biological pathways are present in many organisms, I initially thought that the phenomenon described in reference (1) maybe should be present in many natural sequences as a possible functional common denominator.

I initially supposed that the study of sequences similar or related to the one present in reference (1) maybe could be helpful for our understanding of the molecular basis to biological change.

Thus, this particular phenomenon was a possible prospect for the abundance of proteins exceeding their number of genes via multiple modular combinations of diverse mRNAs. Recent estimates for humans reckon above a million of proteins produced by just 20,000 to 25,000 genes (4).

With these considerations in mind, my initial idea was that, if reference (1) was true, then the production of those numerous proteins could have had a putative process of RNA hetero-linkage at their formation.

However, after five years of comparing sequences, I came to realize that those hetero-sequences using that same oligonucleotide as its common linker were just methodological artifacts.

The common element of these chimeras is the linker **ccgaattcgg** (as presented in reference 1 inside the sequence L21934 for the *H. sapiens* ACAT-1 enzyme). This leaves references 1, 5 and 6 (if real), as a one and unique possible *species-specific* phenomenon in humans (2, 3).

Another article on the same sequence (1), has recently been published by the same group (5). Its authors mentioned since reference (1) the similarity of that linker with the *EcoRI*-adapter (a tool extensively used in molecular biology research), so the door still is open to verify whether this is a methodological artifact or not (5).

The initial construction of that sequence demonstrates that their cDNA library was transformed in *E. coli* (strain MC1061) using the phagemid vector *pBluescript* as well as with the expression vector *pcDNA*. Then, they retransformed it in the same *E. coli* strain (6), again. However, I have recently seen that the use of similar vectors can be involved in the production of chimerical artifacts in multiple instances, like in those examples presented in Tables 1 and 2 (7).

A possible, however remote, explanation for reference (1) is that we are dealing with a natural process, mostly restricted to humans. Yet, whatever the final verdict may be, the fact is that the *EcoRI*-like linker or adapter described in (1) was the starting point for the next findings, described in this article.

My personal hypothesis is that heterotranscripts or chimeras including the *EcoRI*-related palindromic linker **ctcgtgccgaattcggcaccag** or its related sequences, extending themselves to at least twelve bases, are artifacts from the molecular methodologies used, mainly mediated by its host-vector interactions.

## RESULTS AND DISCUSSION

### The finding of a related palindrome in Affymetrix microarrays

The basis for this article appeared while working with antiobesity microarrays. By studying the changes of gene expression in the obesity resistant perilipin knock out mice (8, 11), with the DNA-Chip *Affymetrix* MG-U74A-v2, analyzed using the free educational software *dChip* V.1.2 (9). One particularly intriguing hetero-transcript was the nucleotide sequence AB030505, initially reported by its submitters as the *Mus musculus* mRNA for UBE-1c1, UBE-1c2 & UBE-1c3 (complete cds). The following paragraph describes the sequence AB030505 and the common *EcoRI*-like linking element present in thousands of other *Genbank* sequences.

A careful study of the nucleotide sequence AB030505 using *Blast* (10) led me to an element that was linking two large sections from two different genes:

1. The nucleotide sequence AK078792 from chromosome 10, coding for a melanoma ubiquitous mutated protein homologue (Mum1) and
2. The nucleotide sequence BC036273 from chromosome 12, coding for retinol dehydrogenase 11 (similar to Arsdrl). The linking element within the sequence AB030505 corresponded to the palindrome *ctcgtgccgaattcggcag*, composed by 22 nucleotides.

Here again, as in the initial report (1), two transcripts originated in two different chromosomes were linked together in one mRNA strand. Those 22 bases contain the core palindromic linker *ccgaattcgg* at its center, which is similar to the one initially reported by reference (1).

A palindrome sequence for the double helix of DNA has the same nucleotides if read from 5' to 3', which is the normal reading direction, either from the plus (+) or from the minus (-) strand. A manual and visual assessment of this palindromic linker was done. Amazingly, this linker was present in thousands of sequences reported to the *Genbank*.

In the full Table 2 (7), I present many examples of the palindrome (or related sequences) being reported as if they were present inside coding regions. The palindromic linker mentioned is frequently translated as the artificial peptide **RAEFGT**, absent in sequenced protein databases (10).

### **Increase in the number of palindromic sequences reported to the *Genbank***

A monthly increase was seen in the number of sequences containing the *EcoRI*-like linker or its derivatives inside thousands of sequences. In one recent example (14 Oct. 2005) done in *Blastn* (nucleotide to nucleotide alignments), selecting the *non redundant* (*nr*) nucleic acid database sequences of *Genbank*, a query of 44 palindrome letters was used:

**CTCGTGCCGAATTCGGCACGAGCTCGTGCCGAATTCGGCACGAG**

With this query, I obtained 6010 Blast Hits using the next query conditions:

1.  $10^6$  as the minimum expected number. Some results are presented in Table 2 (7).
2. 1000 as the number of descriptions and of alignments.

In the *Genbank's* alternate database containing *expressed sequence tags* (*est*), which are mRNAs for putative proteins, the number of sequences containing the palindromic *EcoRI*-adapter is also present by the thousands.

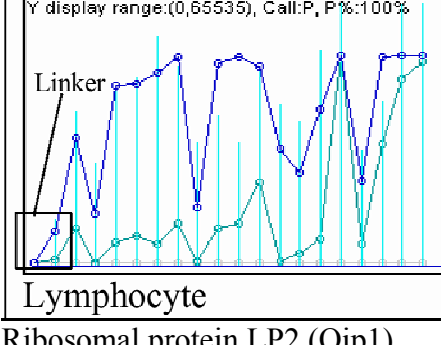
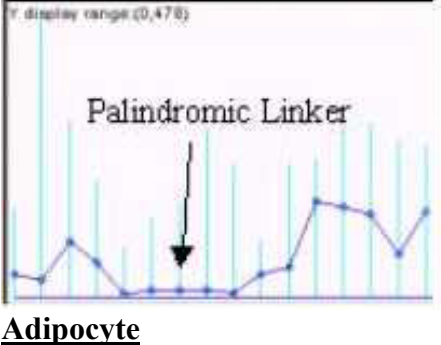
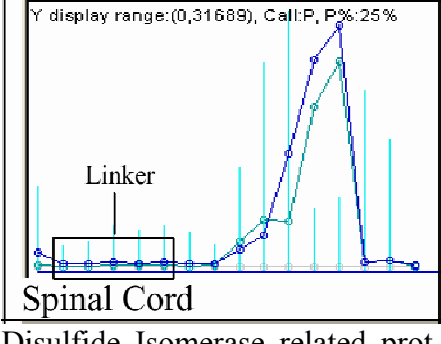
### **Additional palindromes found by using microarrays**

Additional targets pertaining to these linkers were also found while studying the results of microarrays available *online* using the software tool *dChip* (9) coupled to the *Affymetrix'* probes databases. Table 1 shows examples containing the palindromic linkers.

*Affymetrix* has been a successful microarray methodology, *i.e.*, to evaluate the gene expression in humans, mice, and rats. However, both the presence of artificial heterotranscripts and/or of their

own artificial linkers can lead to a misrepresentation of its real expression inside the tissues, as the area under the curve is reduced for those genetic sequences.

**Table 1.** The *EcoRI*-related palindromic linker is present both in the *Genbank* sequence targets and in *Affymetrix* microarray probes for humans, mice and rats.

ID/ Organism	<i>EcoRI</i> Affymetrix Probes from Genbank [DNA Chip]	Graphic, non-expression of <i>EcoRI</i> -linker	Reference
AB002533_at  <i>Homo sapiens</i>	gaattcggcagagcacgcgtgaga, ggcagagcacgcgtgagacttctc  [in the Human DNA Chips <i>HuGene-FL</i> and in <i>Hu6800</i> ]		Shipp <i>et al</i> , <i>Nature Med.</i> 8, 68. 2002
AJ243503  (99534_at)  <i>Mus musculus</i>	ttcggcagagctcgtgccgtcct  [in the Mouse DNA Chips <i>MG-U74Av2</i> and in <i>MG-U74A</i> ]		Castro-Chavez <i>et al</i> . <i>Diabetes</i> 52, 2666. 2003
AI045710  (rc_AI045710_at)  <i>Rattus norvegicus</i>	atgatgtacagatccctcgtgcc, tgatgtacagatccctcgtgcc, tatgtacagatccctcgtgccct, tgtacagatccctcgtgccctcg, gtacagatccctcgtgccctcgt  [in the Rat DNA Chips <i>RG-U34B</i> ]		Children's National Medical Center [ <a href="http://pgadata.cnmcresearch.org/spinalcord.asp">http://pgadata.cnmcresearch.org/spinalcord.asp</a> ]. Accessed Feb. 1, 2005.

**Note:** The *EcoRI*-related palindromic linker **ctcgtgccgaattcggcagag** causes the drop of microarray expression to zero demonstrating its absence in the tissues [*dChip V.1.2* (9)]. Highlighted in the second column in clear blue are the portions corresponding to the palindromic linker, and in dark blue, the nucleotides exchanged to obtain the second set or "mismatch" in *Affymetrix*' probes (*DNA-Chip*).

## The phenomenon of heterotranscription

Twelve bases seem to be the minimum common denominator in order for the *EcoRI* palindromic linker to produce artificial heterotranscripts such as the ones reported here and present in the *Genbank*.

The most common palindromic flanks for the oligonucleotide **ccgaattcgg** are **g** and **c**, which give us the longer oligonucleotide **gccgaattcggc**. Less frequent are the flanks **c** and **g** to produce the second oligonucleotide **cccgaattcggg**, with a similar effect on heterotranscription. This last palindromic sequence is the one that we have in reference (1). The same palindromic sequence is present in example 9 from Table 2 (*Homo sapiens* X93499 for the RAB7 protein), in which we have fragments for more than two genes attached together in the same strand, through the palindromic linkers **ccccgaattcgggg** and **gccgaattcgggc** (12).

**Table 2.** Transcripts with traces of an *EcoRI* related linker and some related references.

#	Accession / Organism	Gene/Protein (Gene symbol) [notes for sides of linker (L or R)]	Linker and its Translation in Amino Acids as presented in the <i>Genbank</i>	Corresponding References According to the <i>Genbank</i> ; closest related match
1	U58090 <i>Homo sapiens</i>	Cullin gene family member, Hs-cul-4A	aatcggcagcagctcgtgccgct NSARARAA	<i>Cell</i> 85, 829-839. 1996.
2	U28831 <i>Homo sapiens</i>	Protein immuno-reactive with anti-PTH polyclonal antibodies	gcagcagctcgtgccgat ARARAD	<i>Proc. Assoc. Am. Physicians</i> 107, 296-305. 1995.
3	BC041619 <i>Homo sapiens</i>	Protein KIAA0404, for IMAGE:5923662 [R: hypoth. prot. MGC16044]	ccctcgtgccgaattcggcagcag PSCRIRHE	<i>Proc. Natl. Acad. Sci. U.S.A.</i> 99, 16899-16903. 2002.
4	AF176705 <i>Homo sapiens</i>	F-box protein FBX10 (PINX1) [R: vector]	cctcgtgccgaattc PRAEF	<i>Curr. Biol.</i> 9, 1180-2. 1999.
5	X85792 <i>Homo sapiens</i>	Vpr binding protein 1	tcgtgccgaattcggcagcag SCRIRHE	Benichou <i>et al.</i> [Unpublished]; <i>J Biol Chem.</i> 277, 45091-8. 2002
6	AF151109 <i>Homo sapiens</i>	Putative BRCA1-interacting protein (BRIP1)	ggcagcagctcgtgccgc GTSSCR	Wang <i>et al.</i> BRCA1-interacting protein. [Unpublished]; <i>Oncogene</i> 19, 6152-8. 2000.
7	AF146697 <i>Homo sapiens</i>	FOXP1	aagaattcggcagcagct KNSARA	<i>Cancer Res.</i> 61, 8820-8829. 2001.
8	NM_002342 <i>Homo sapiens</i>	Gene and 3' UTR for TNFR superfamily, member 3 (LTBR)	gctcgtgccgaattc	<i>Genomics</i> 16, 214-218. 1993. [Curated by NCBI]

9	X93499 <i>Homo sapiens</i>	RAB7 protein, GTP-binding [L: Dystroglycan 1. C: Rab7. R: Envelope glycoprotein]	<b>ccccgaattcgggg gcccgaattcgggc</b>	& <i>Biochem. Biophys. Res. Commun.</i> 229, 887-890. 1996.
10	X82200 <i>Homo sapiens</i>	Gene and mRNA for interferon-induced Staf50	<b>gaattcggcagagctc</b>	<i>J. Biol. Chem.</i> 270, 14891-14898. 1995.
11	U31384 <i>Homo sapiens</i>	mRNA for G protein gamma-11 subunit	<b>ggcagagctcgtgccg</b>	<i>J. Biol. Chem.</i> 270, 21765-21771. 1995.
12	AF379619 <i>Homo sapiens</i>	Intron near AB13, precursor mRNA	<b>gaattcggcagagct</b>	van Roy and Staes. New human gene family. [Unpublished].
13	AY245868 <i>Homo sapiens</i>	CDS for Aldehyde oxidase-like protein (AOX2) pseudogene	<b>aagaattcggcagagca LNSARA</b>	Wright RM. Human aldehyde oxidase. [Unpublished].
14	AF339764 <i>Homo sapiens</i>	mRNA from Fetal liver spleen IMAGE:108721	<b>gaattcggcagagcggcagagct</b>	<i>Genomics</i> 79, 635-656. 2002.
15	U43527 <i>Homo sapiens</i>	5'UTR for Malignant melanoma metastasis-suppressor (KiSS-1)	<b>(ctct)<sup>15</sup>cctcgtgccgaattcggcagag</b>	<i>J. Natl. Cancer Inst.</i> 88, 1731-1737. 1996; <i>Genomics</i> 54, 145-148. 1998.

**Note:** This table presents the *EcoRI* related linker ***ctcgtgccgaattcggcagag*** as it appears in **the Genbank** for some human genes. To view the rest of this Table 2 and the presence of the linker in other organisms, refer to URL: <http://www.geocities.com/plin9k/t2.htm>

### Abundance of sequences including the *EcoRI*-like palindromic linker

There are thousands of sequences, including *expressed sequence tags (est)* in the *Genbank* and in other nucleotide databases that still contain artifacts, having as its common denominators, *EcoRI* palindromic linkers like the ones described in this article.

The palindromic linkers can be present in tandems, halves, or in different lengths; being 12 to 24 bases its most common range. Artificial linkers have been found even inside multiple coding regions, like the examples presented in the full Table 2 (7). Examples of those linkers are frequently present outside the coding region, *i.e.*, in promoters, like in:

- 1-) NR\_001557 for *H. sapiens* aldehyde oxidase 2 (AOH2) on chromosome 2, oligo ***gaattcggcagagc*** (13).
- 2-) NM\_002342 *H. sapiens* lymphotoxin beta-receptor (LTBR; member 3 of the TNFR superfamily), oligo ***gctcgtgccgaattc*** (14).

Furthermore, those palindromic linkers have been found also in the 5' region, *i.e.*, in sequence U43527 for the human malignant melanoma metastasis-suppressor KiSS-1, oligo ***(ctct)<sup>15</sup>cctcgtgccgaattcggcagag*** (15), and/or in the 3' region, *i.e.*, sequence AY029161 for the Pin2-interacting protein X1, oligo ***ctcgtgccgaattcggcac*** (16).

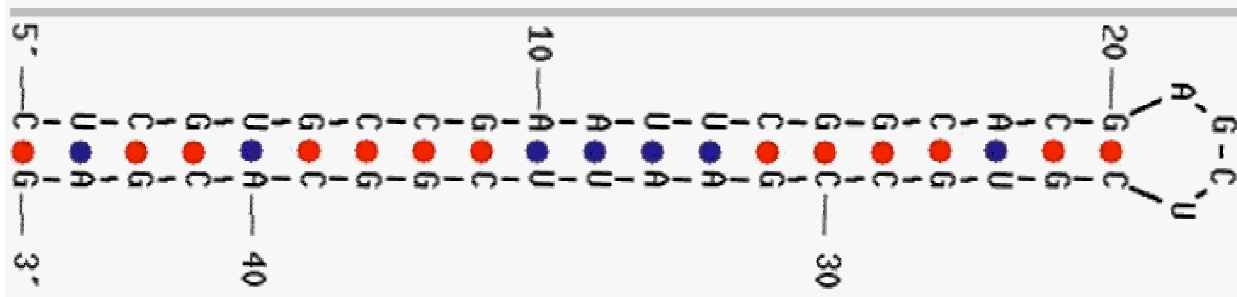
Of the few submissions to the *Genbank* that are explicitly reporting the presence of the *EcoRI* adapter, Hirama *et al* (17) stands out, together with Inoue *et al* (18) and Savas *et al* (19). However, Inoue *et al* (18) considers only the 8 first bases at the left flank as part of the sequence for the *EcoRI*-adapter. The effect of the palindromic linker for Inoue's sequence may extend to at least 16 bases (sequence D83948 for S1-1 protein, oligo ***ggcacgagctcgtgccg***) by an apparent phenomenon of self-recombination and self-insertion inside the host-vector interactions.

A similar situation to what we see in Inoue's is presented by Savas *et al* (19), mentioning in their *Genbank* submission the first 6 bases only as part of the *EcoRI*-adapter (same in reference 1, but not in the submitted sequence L21934). In Savas' reference (19) the linker-like effect may be extended about 20 bases (sequence X78445 for Cytochrome P450 Cyp1-b-1, oligo ***gaattcggcaccgaactcgtgc***). Hirama *et al* (17) is the only one that appropriately mentions a longer extension for the *EcoRI*-adapter, reporting it as being of 14 bases (sequence X56703 for the rearranged T-cell receptor alpha chain, oligo ***gaattcggcaccgagct***).

### Chimaeras linked by the *EcoRI*-like palindrome seem to be resistant to enzymatic digestion

The palindromic linkers persist in the sequences without being digested by the enzymes. The discovery of mechanisms of resistance to the enzymatic digestion awaits further study. However, it is evident that the most common palindromic linkers match the identity of the *EcoRI* adapter sequence ***gaattcggcaccgag***, which is used for the 5'UTR, and reported to the *Genbank*, *i.e.*, inside the sequence A1607511 as ***ctcgtgccgaattcggcaccgag*** (similar to acidic ribosomal phosphoprotein PO). In that sequence, it is indicated the use of the vector *pBluescript* SK(-), plus *EcoRI*, with the additional use of the vector *Uni-ZAP XR*. These methodologies, like the ones described in (1, 5, 6), may be promoting the phenomenon of artificial linkage abundantly present in the *Genbank*.

The rearrangement and splicing in a host-vector interaction resistant to *EcoRI* enzymatic digestion may be explained by a phenomenon of self-hybridization performed by the linker (Figure 3), which could make it to appear as an appendage impossible to be grasped by the digestive enzyme.



**Figure 3.** Self-hybridization of the palindromic *EcoRI*-like linker seems to block enzymatic digestion. Phenomenon also seen as 'a closing zipper', at both ends of longer sequences, where two distant parts of the linker approach and stick together producing a plasmid-like formation. [Examples 38-42, full Table 2 (7). Figure obtained using the software from reference 20.]

### CONCLUSIONS AND PERSPECTIVES

## **Stringent quality control on sequence databases is required**

These findings may contribute to a more stringent implementation of a quality control within nucleotide databases, as well as the professional analysts of molecular artifacts and related experiments, not to mention the rational human engineering of pharmaceutical drugs and proteins. We must first tame, through its controlled use, those palindromic linkers. Then, the use of sequence quality control (to detect those molecular artifacts) can successfully be applied. Improvements can be made in the design of *Affymetrix* microarrays, *i.e.*, by removing the palindromic linker from human sequences such as AA557228, AA113291, AI798671, AA864645, AA780435, W90032, AA810599, T52176, AA976510, AI380906, AA535275, AI792166, T67559, L04270, U83598, D59474, T03148, T54342, D59674, D59787, D80164, H90908, N80129, C14426, D59619, D80240, T56800, C14298, W72424, IR1056496, T69555, D80337, C14227, C14407, C14344, D80210, etcetera.

## **Possible use of palindromes and its heterotranscripts to reverse hereditary diseases**

Another possibility is the use of palindromes for the attachment of two different genes (genetic modules) for the engineering of therapeutic proteins, *i.e.*, for the engineering of antiobesity treatments that may be customized and prescribed according to the particular metabolism of each person (21).

## **Artificial vectors for gene therapy may produce heterotranscription**

It is possible that palindromic linkers like the ones reported in this article, or using other linkers, may be produced by artificial vectors for gene therapy also. If this is the case, an unsuspected side effect for humans may result. To date, those linkers are still present in those thousands of artificial sequences submitted to the *Genbank*, and are an allegory of things that can be prevented in human gene therapy. This article has been written to raise the awareness to the scientific community on the presence of these thousands of artificial hetero-transcripts by current methodologies producing the sequences reported to the *Genbank* as well as the vectors used in gene therapy.

## **Published and submitted heterotranscripts are still awaiting its correction**

In doing an analysis of the sequences presented as chimerical mRNAs in Table 4 of reference (22), I also found in that article the *EcoRI*-related palindromic linker present inside the sequence AY029161 (16), already seen. In that sequence, there is a fragment for a putative human tumor suppressor LPTL, AF418553, originated in the human chromosome 8, and linked with a fragment for phosphohistidine phosphatase PHP14, NM\_014172 originated in the human chromosome 9.

Until the submission of this article, none of the sequences presented here had been corrected in the *Genbank*. Could this be because they have not been determined as artifact products to date?

Another sequence in reference (22) that contained the *EcoRI* related palindromic linker was BC000519, identified as an artifact and has already been removed from the *Genbank*.

### **A possible transposon-like removal of palindromic linkers seem to have happened on other chimeras**

Awaiting correction are thousands of sequences like the ones presented in Table 2 (7). Sequences like AF176705 for the human F-box protein FBX10 (23), and Z28355 from the atrium heart (24), are both containing fragments of the vector and of the linker. Another sequence, HTCBYB08, contains fragments of the vector only, lacking the linker. Experiments were also done with such sequences lacking any evidence of a palindromic linker, also without obtaining amplified products by RT-PCRs (*unpublished results*). Both of the sequences Z28355 and HTCBYB08 exhibit traces of the same cloning vector, vector from the sequence X52324 (ARBLSKM), the *pBluescript SK(-)* vector (25-27). A transposon-like self-removal of the palindromic linker may be one way in which many heterotranscripts appear to be lacking the linker, as in the sequence HTCBYB08. This hypothesis also needs to be experimentally evaluated. If this is the case, palindromic linkers may be used for a possible artificial reversal of mutations *in vivo* (28).

On the other hand, heterotranscripts lacking the linker, and present in *Affymetrix'* microarrays, may show two different expression profiles, for example, a zero expression on one side while a contrasting higher expression on the other side (*data not shown*). A similar pattern is present when non-expressed introns are included within *Affymetrix'* probes side by side with sequences expressed by the exons, as in Figure 1 from reference (2).

### **Final comment**

The key palindrome described in this article has already been introduced in reference (2) as **CTCGTGCCGAATTCGGCACGAG** and it has been reported elsewhere (2, 3, 29), also.

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