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Aberrant allele-switch imprinting of a novel *IGF1R* intragenic antisense non-coding RNA in breast cancers

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Abstract The insulin-like growth factor type I receptor (*IGF1R*) is frequently dysregulated in breast cancers, yet the molecular mechanisms are unknown. A novel intragenic long non-coding RNA (lncRNA) *IRAIN* within the *IGF1R* locus has been recently identified in haematopoietic malignancies using RNA-guided chromatin conformation capture (R3C). In breast cancer tissues, we found that *IRAIN* lncRNA was transcribed from an intronic promoter in an antisense direction as compared to the *IGF1R* coding mRNA. Unlike the *IGF1R* coding RNA, this non-coding RNA was imprinted, with monoallelic expression from the paternal allele. In breast cancer tissues that were informative for single nucleotide polymorphism (SNP) rs8034564, there was an imbalanced expression of the two parental alleles, where the 'G' genotype was favorably imprinted over the 'A' genotype. In breast cancer patients, *IRAIN* was aberrantly imprinted in both tumours and peripheral blood leucocytes, exhibiting a pattern of allele-switch: the allele expressed in normal tissues was inactivated and the normally imprinted allele was expressed. Epigenetic analysis revealed that there was extensive DNA demethylation of CpG islands in the gene promoter. These data identify *IRAIN* lncRNA as a novel imprinted gene that is aberrantly regulated in breast cancer.

1. Introduction

Despite recent advances in molecular therapeutics, breast cancer remains a highly lethal malignancy worldwide [1]. Anti-human epidermal growth factor receptor 2 (HER2) antibody therapy using Herceptin has been successful in the treatment of HER2-positive early stage

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and metastatic breast cancers [2,3]. However, resistance to Herceptin therapy has become an obstacle for treatment of HER2-positive breast cancer patients [4,5]. The activation of alternative growth factor pathways, particularly via the insulin-like growth factor 1 receptor (*IGF1R*), represents a common feature of Herceptin-refractory cells [6].

IGF1R is one of the most abundantly phosphorylated receptor tyrosine kinases in tumours [7–9]. The insulin-like growth factor system, including the type I IGF receptor *IGF1R* and the mitogenic ligands IGF-I and IGF-II, is frequently dysregulated in breast cancer and is known to contribute to disease progression and metastasis [10–14]. IGF-I and IGF-II promote cell growth and survival via the IGF1R receptor-mediated signal transduction through intracellular tyrosine kinase linked to the phosphatidyl-inositol-3 kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway. Overexpression of *IGF1R* activates the PI3-K and mitogen-activated protein kinases (MAPK) signal cascades, resulting in cell proliferation and resistance to chemotherapeutic agents, radiation, and targeted therapies using Tamoxifen and Herceptin [15–17]. Therapeutic agents targeting *IGF1R* are currently in clinical development [10–14,18–23], including those that inhibit the IGF1R tyrosine kinase using monoclonal antibodies and small molecules [24]. However, the clinical development of various IGF1R inhibitors has been put on hold due to lack of sufficient clinical efficacy. Thus, the regulation of this pathway needs to be further defined to aid in the development of next generation regimens.

Currently, the molecular mechanisms underlying the dysregulation of the *IGF1R* pathway in tumours remain unknown. Using a recently-developed R3C (**R**NA-guided **C**hromatin **C**onformation **C**apture) technique [25], we recently identified a novel long non-coding RNA (lncRNA) *IRAIN* within the *IGF1R* locus [26]. *IRAIN* is transcribed from an intragenic promoter located in the first intron of *IGF1R*. *IRAIN* lncRNA is transcribed in an antisense orientation compared with the IGF1R gene, and it is expressed exclusively from the paternal allele, with the maternal allele being silenced. Interestingly, this lncRNA interacts with chromatin DNA and is involved in the formation of an intrachromosomal enhancer/promoter loop. In addition, *IRAIN* was downregulated in leukaemia cell lines and in leucocytes from patients with high-risk AML [26]. These data suggested that *IRAIN* might play a role in the dysregulation of the IGF pathway in haematopoietic malignancies.

However, the function of this non-coding RNA in other malignancies remains to be explored. The *IGF1R* pathway is frequently dysregulated in breast cancer. It is unclear if *IRAIN* lncRNA is aberrantly imprinted in breast cancer patients. In this communication, we characterise the allelic expression of *IRAIN* lncRNA in a cohort of breast cancer samples.

2. Materials and methods

2.1. Breast cancer cell lines and tissues

Breast cancer cell lines (MCF7 and MDA-MB-231) used in this study were purchased from ATCC. Cells were grown in RP1640 Media, supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin.

Breast tumour specimens were collected from 74 female patients with invasive breast cancer who were treated at The First Hospital of Jilin University between 2007 and 2010. All tumour samples were obtained from patients with invasive ductal carcinomas (IDC) ($n = 74$) (Table S1). Normal breast tissues ($n = 9$) were collected as control samples from the patients who either underwent prophylactic mastectomy ($n = 3$) or in whom normal breast tissue was removed at a site distant from the primary tumour ($n = 6$). The protocol was approved by the Human Medical Ethical Review Committee from Jilin University First Hospital and informed consent was obtained from each breast cancer patient and normal subject.

Samples were snap frozen in liquid nitrogen at the time of the pre-therapeutic biopsy or surgical treatment and were stored at –80 °C for total RNA and genomic DNA extraction. The pathological diagnosis was made in accordance with the histological classification of tumours developed by the World Health Organisation. Tumour stage was defined according to American Joint Committee on Cancer/International Union Against Cancer tumour, node, metastasis (TNM) classification system. Tumours were histologically graded according to the Elston and Ellis method. Molecular markers, including the oestrogen receptor (ER), the progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and the mitotic index Ki67, were examined by using immunohistochemical (IHC) methods. Patients with Her2/neu2$^+$ were tested for gene amplification using fluorescence in situ hybridisation (FISH) for validation. Breast cancer molecular subtype was defined by IHC receptor status of breast cancer according to St Gallen International Breast Cancer Conference (2013). We divided the patients into three groups: (1) Triple negative breast cancer (TNB, ER$^-$, PR$^-$, HER2$^-$), (2) HER2$^+$ (ER$^-$, PR$^-$, and HER2$^+$), (3) luminal ['Luminal A-like': ER$^+$ and PR$^+$ ($\geqslant$20%), HER2$^-$, Ki67 < 14%; 'Luminal B-like (HER2 negative)': ER$^+$, HER2$^-$, and at least one of: Ki-67 $\geqslant$14%, PR$^-$, PR$^+$ (<20%); 'Luminal B-like (HER2 positive)': ER$^+$, HER2$^+$, Any Ki-67, Any PR)]. After pathologic diagnosis, patients were treated according to standard clinical protocols. Clinical data such as date of birth, sex, date of surgery were extracted from the computerised clinical database.

Peripheral blood leucocytes (PBL) collected from breast cancer patients were isolated by Ficoll-Hypaque

(Sigma, MO) centrifugation and then cryopreserved for DNA and RNA analyses.

2.2. Reverse transcription-polymerase chain reaction (PCR) analysis

As previously described [25,27], total RNA was extracted from tissues by TRI-REAGENT (Sigma, CA) and cDNA was synthesised with RNA reverse transcriptase. Briefly, 1 μg of total RNA was used, and PCR was carried out under liquid wax in a 6 μl reaction mixture containing 2 μl of 3 × Klen-T*aq* I Mix, 2 μl cDNA and 1 μl of each 2.5 μM primer. After incubation at 95 °C for 2 min, *IRAIN* cDNA was amplified by 32 cycles of 95 °C for 30 s, 65 °C for 30 s of annealing and 72 °C for 35 s of extension, and finally with extension at 72 °C for 5 min.

For real-time quantitative polymerase chain reaction (qPCR), cDNA samples were amplified using CFX96™ real-time system (BIO-RAD) by SYBR PrimeScript™ RT-PCR Kit (Takara, Japan). The mRNA expression level of *IRAIN* and *IGF1R* was quantitated by normalising with β-actin (housekeeping gene) as previously described [27,28]. PCR primers used for qPCR and RT-CPR are listed in Table S2.

2.3. Gene strand-specific RT-PCR

The orientation of *IRAIN* was mapped with a strand-specific PCR (SSRT) assay [26,27]. Total RNA was extracted from tissues by TRI-REAGENT (Sigma, MA). Total RNA (400 ng) was reverse transcribed with the *IRAIN* 5′- or 3′-primers using Maxima Reverse Transcriptase (Thermo Fisher Scientific, CA) at 60 °C for 50 min, followed by 85 °C for 5 min to inactivate the transcriptase. After 10-fold dilution, PCR was carried out under liquid wax in a 6 μl reaction containing 2 μl of 3 × Klen-T*aq* I Mix, 2 μl cDNA and 1 μl of each 2.5 μM downstream PCR primer set. After initial denaturing at 95 °C for 2 min, *IRAIN* cDNA was amplified by 32 cycles at 95 °C for 30 s, 65 °C for 30 s of annealing and 72 °C for 35s of extension, followed by incubation at 72 °C for 5 min. PCR primers used for strand-specific PCR are listed in Table S2.

2.4. Allelic expression

Quantitation of allelic expression requires the presence of heterozygous single nucleotide polymorphisms (SNPs) to distinguish the two parental alleles. We extracted genomic DNAs from breast cancer samples and screened for heterozygosity of SNPs in *IRAIN* lncRNA [26]. Only those SNP-informative breast cancer samples were used for *IRAIN* imprinting analysis. However, for imprinting assessment, the data of allelic distribution of the parents were needed to track which parental allele was expressed. In these studies, only those cases with parental information available were included. To examine differential allelic expression of *IRAIN* lncRNA between tumours and peripheral blood leucocytes (PBL), only those informative cohort cases with available blood samples were included in the study.

Total RNA extraction and cDNA synthesis were performed as previously described [29,30]. Allelic expression of *IRAIN* was examined using the same program as in the RT-PCR but using primers specific for polymorphic restriction enzymes. Allelic expression of *IRAIN* was assessed by polymorphic restriction enzymes Nde1. In some cases, DNA sequencing of genomic DNA and cDNA PCR products was used to determine allelic expression of *IRAIN*. PCR primers used to assess allelic expression covering the Nde1 polymorphic site were JH891 and JH892, and primers for allelic sequencing using SNP rs8034564 were JH248 and JH781 (listed in Table S2).

2.5. DNA methylation analysis

Genomic DNA was extracted from cells and tumours with Perfect gDNA kit (Eppendorf, NY). Genomic DNA (1 μg) was used for bisulphite conversion with EZ DNA Methylation-Gold™ Kit (ZYMO Research, CA) according to the manufacturer's instructions. PCR reactions were performed using Kantaq1 DNA polymerase (Ab Peptides, MO). Bisulphite-sequencing PCR (BSP) was used to analyse DNA methylation status. PCR conditions were 97 °C for 10 min followed by 35 cycles of 96 °C for 20 s, 64 °C for 30 s of annealing, 72 °C for 30 s of extension, and completing the reaction at 72 °C for 10 min. The primers used for assessing DNA methylation were JH852 and JH855 (Table S2) [26]. PCR products were separated by gel electrophoresis and purified with Axygen DNA Gel Extraction kit (Axygen, CA). The PCR products were cloned into CloneJET vector using PCR Cloning Kit (Thermo Scientific # K1231, MA), and sequenced for analysis of CpG methylation.

2.6. Statistical analysis

All experiments were performed in triplicate, and the data are expressed as mean ± SD. The data were analysed by one-way analysis of variance, and results were considered statistically significant at $P \leqslant 0.05$.

3. Results

3.1. Ubiquitous expression of IGF1R intragenic non-coding RNA in breast cancers

In studying the tumour-specific dysregulation of *IGF1R*, we recently identified a novel 5366 bp *IGF1R*-intragenic long non-coding RNA (*IRAIN*) that is associated with haematopoietic malignancies [26]. To

determine if this non-coding RNA is aberrantly regulated in breast cancer, we first used RT-PCR to determine the presence of this non-coding RNA in breast cancer tissues. We found that *IRAIN* lncRNA was ubiquitously expressed at various levels in the breast cancer samples (Fig. 1A).

We then grouped the breast cancer patients into three groups: (1) Triple-negative breast cancer, (2) $HER2^+$ and (3) Luminal (luminal A-like, luminal B1-like, luminal B2-like) as described in Section 2. As shown in Fig. 1B, *IRAIN* lncRNA was downregulated in both TNB and $HER2^+$ groups ($P < 0.05$).

3.2. *IRAIN* is transcribed antisense to *IGF1R* in breast cancers

We then used a strand-specific RT-PCR (SSRT) method to map the orientation of gene transcription. SSRT cDNA was synthesised by Thermo-stable reverse transcriptase utilising a 5′-specific oligonucleotide or a 3′-specific oligonucleotide, respectively. After SSRT, a pair of downstream PCR primers was used to amplify the strand-specific cDNA (Fig. 2A).

As seen in Fig. 2B, *IRAIN* RNA was detected only when cDNA was synthesised using 5′-oligonucleotides (#513, #400) (lanes 1, 4, 7, 10). No PCR products were amplified when the 3′ oligonucleotides were used (#514, #401, lanes 2, 5, 8, 11) or in the RT-minus controls (lanes 3, 6, 9, 12), indicating that *IRAIN* was transcribed in the antisense direction as compared with the *IGF1R* coding RNA.

3.3. Monoallelic expression of *IRAIN* in breast cancer tissues

In the mouse, the gene transcribing the Type 2 IGF receptor (*Igf2r*) is associated with an lncRNA *Airn* that is transcribed antisense to *Igf2r*. These transcripts are reciprocally imprinted, with *Airn* transcribed from the paternal allele only. The transcription of the antisense lncRNA *Airn* regulates in *cis* the allelic expression of the *Igf2r* coding RNA [31–34]. In leukaemia cells, we showed that *IRAIN* was expressed solely from the paternal allele [26]. To learn if *IRAIN* uses a similar epigenetic mechanism to regulate genes locally in breast cancers, we examined if *IRAIN* lncRNA was monoallelically expressed in the MCF7 breast cancer cell line, which is heterozygous for the polymorphic Nde1 restriction site. Two alleles, termed 'A' and 'G', were detected in genomic DNA (Fig. 3A, lanes 2–3). In cDNA samples, however, only the 'A' allele was detected (lanes 5, 6). The other parental allele (G), in contrast, was totally suppressed. These data indicate that *IRAIN* lncRNA is monoallelically transcribed in the MCF7 breast cancer cell line.

We then examined the allelic expression of *IRAIN* lncRNA in breast cancer tissue samples using SNP rs8034564 to distinguish the two parental alleles. As this SNP does not contain a restriction enzyme site, PCR sequencing was used to determine the allelic expression of *IRAIN*. In three breast cancer tissues that were heterogeneous for this SNP, both the 'A' and 'G' alleles were observed in genomic DNAs (gDNA). However, in all cDNA samples tested, only a single parental allele (A)

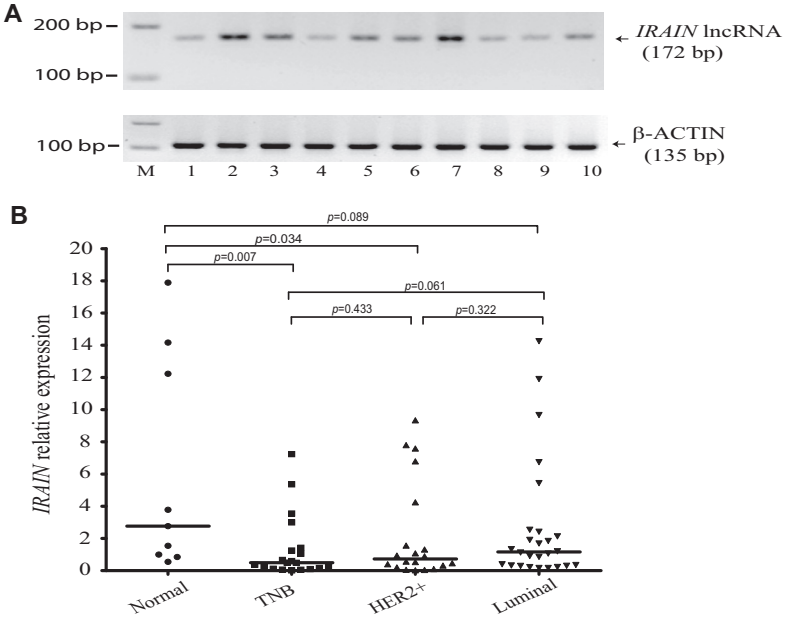

Fig. 1. Downregulation of *IRAIN* long non-coding RNA (lncRNA) in breast cancer. (A) Ubiquitous expression of *IRAIN* in breast cancer tissues. Expression of *IRAIN* lncRNA was analysed by reverse transcription-polymerase chain reaction (RT-PCR). β-Actin was used as the internal control. Lanes 1–4: HER2+; lanes 5–7: Luminal; lanes 8–10: TNB. (B) Dysregulation of *IRAIN* lncRNA in breast cancer subtypes. TNB, Triple-negative breast cancer (TNB, ER^-, PR^-, $HER2^-$); $HER2^+$: (ER^-, PR^-, and $HER2^+$); Luminal: ['Luminal A-like': ER^+ and PR^+ ($\geqslant$20%), $HER2^-$, Ki67 <14%; 'Luminal B-like (HER2 negative)': ER^+, $HER2^-$, and at least one of: Ki-67 $\geqslant$14%, PR^-, PR^+ (<20%); 'Luminal B-like (HER2 positive)': ER^+, $HER2^+$, Any Ki-67, Any PR)]. $^*p < 0.05$ between the groups.

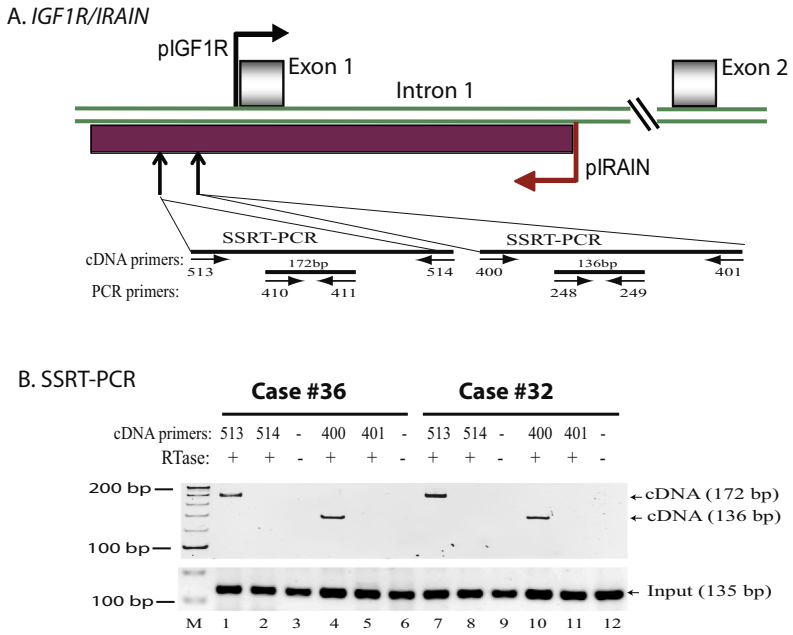

Fig. 2. *IRAIN* is an antisense long non-coding RNA (lncRNA). (A) Diagram of the *IRAIN*/*IGF1R* locus. pIRAIN: *IRAIN* lncRNA promoter that is transcribed in antisense; pIGF1R: *IGF1R* coding RNA promoter that is transcribed in sense. Horizontal arrows: SSRT-PCR primers used to map the orientation of *IRAIN* lncRNA. (B) *IRAIN* lncRNA is an antisense lncRNA. The strand-specific cDNAs were synthesised using either the 5′- or the 3′-oligonucleotides. A pair of polymerase chain reaction (PCR) primers located between two cDNA oligonucleotides was then used to determine the transcription orientation of the *IRAIN* lncRNA. M: 100 bp marker; input: total RNA collected before SSRT-PCR.

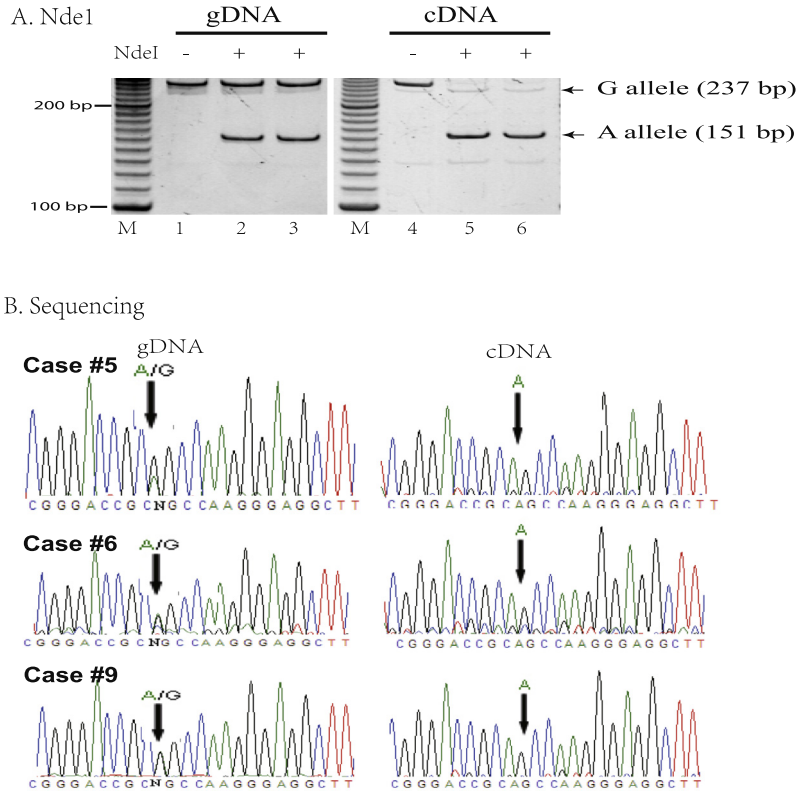

Fig. 3. Monoallelic expression of *IRAIN* long non-coding RNA (lncRNA). (A) Monoallelic expression of *IRAIN* lncRNA in MCF7 breast cancer cell line. gDNA: heterozygous genomic DNA. Note only the single 'A' allele of *IRAIN* lncRNA was detected in cDNA samples. (B) Monoallelic expression of *IRAIN* lncRNA in breast cancer tissues. In breast cancers that are heterogeneous for the *IRAIN* polymorphic site in genomic DNA, only the 'A' allele was expressed. (C) Parental imprinting of *IRAIN* lncRNA. Genomic DNA and cDNA from peripheral blood leucocytes (PBL) were amplified and PCR products were sequenced for the A/G alleles. Note the *IRAIN* lncRNA was expressed from the paternal allele.

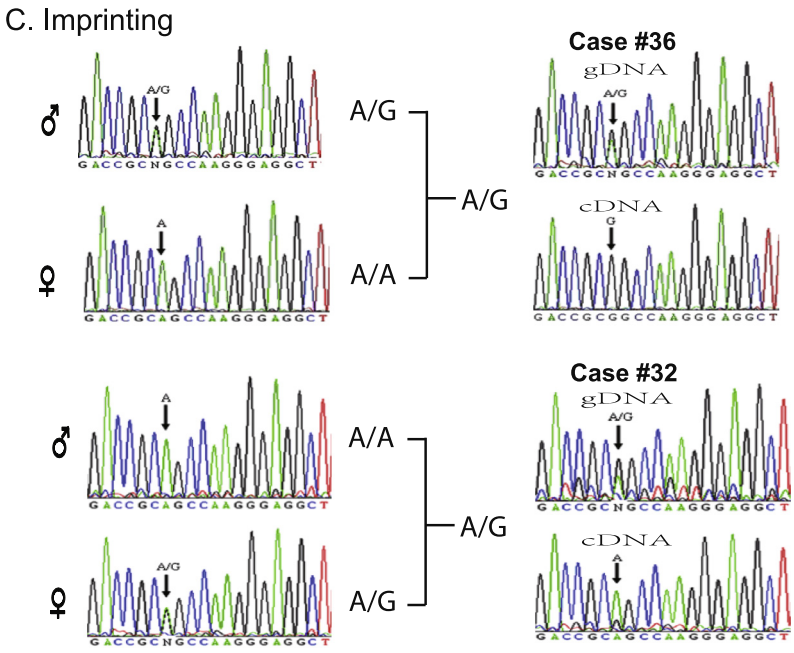

Fig 3. *(continued)*

was detected (Fig. 3B), suggesting that *IRAIN* is mono-allelically expressed in breast cancer.

In examining allelic expression, it was interesting to note the expression of the 'A' allele was favored over the 'G' allele. In 18 breast cancers that were heterozygous for the polymorphic SNP, 16 tumour cDNAs expressed the 'A' allele alone (Table 1).

3.4. *IRAIN* is imprinted in breast cancer tissues

To determine if *IRAIN* is imprinted, we tracked the expression from the paternal or maternal allele using peripheral blood leucocytes from two patients whose parents had also donated blood samples. In Case #36, the father was heterozygous for the A and G alleles while the mother was homozygous for the A allele. The patient was informative at the polymorphic site, carrying both the A and G alleles in the genomic DNA. In the cDNA sample, however, we detected the expression of *IRAIN* lncRNA only from the G allele that was inherited from the father (Fig. 3C, left panel), demonstrating that *IRAIN* is paternally expressed and maternally suppressed. We also confirmed the paternal expression in Case #32. The patient was heterozygous for the A and G alleles. In the cDNA sample, only the paternal A allele was expressed (right panel). Thus, *IRAIN* is maternally imprinted, in agreement with our previous finding in leukaemia samples [26].

Table 1
The favored 'A' monoallelic expression of *IRAIN* in breast cancer tissues.

Case	DNA genotype	cDNA allelic expression	
		G	A
5	AG	–	+
6	AG	–	+
9	AG	–	+
11	AG	–	+
12	AG	–	+
14	AG	–	+
16	AG	–	+
18	AG	–	+
19	AG	–	+
23	AG	–	+
28	AG	–	+
29	AG	–	+
32	AG	–	+
36	AG	+	–
37	AG	–	+
39	AG	–	+
42	AG	+	–
43	AG	–	+
Total		2 (11%)	16 (89%)

3.5. *DNA methylation in the IRAIN promoter*

The *IRAIN* promoter is very rich in CpG dinucleotides. In peripheral blood leucocytes, the promoter CpG islands were semi-methylated [26]. We analysed the status of DNA methylation in the *IRAIN* promoter in our breast cancer specimens and cell lines (Fig. 4A). Using bisulphite sequencing, we found that the *IRAIN* promoter is totally unmethylated in two breast cancer cell lines (MCF-7, MDA-MB-231) (Fig. 4B).

We also examined DNA methylation in three breast cancer tissues that show monoallelic expression. In the breast cancer specimens, we observed a hemi-methylation pattern in the *IRAIN* promoter in two breast cancer samples (Cases #5, #9) (Fig. 4C). However, in Case #6, the *IRAIN* promoter was almost totally unmethylated, as was seen in the two breast cancer cell lines. Thus,

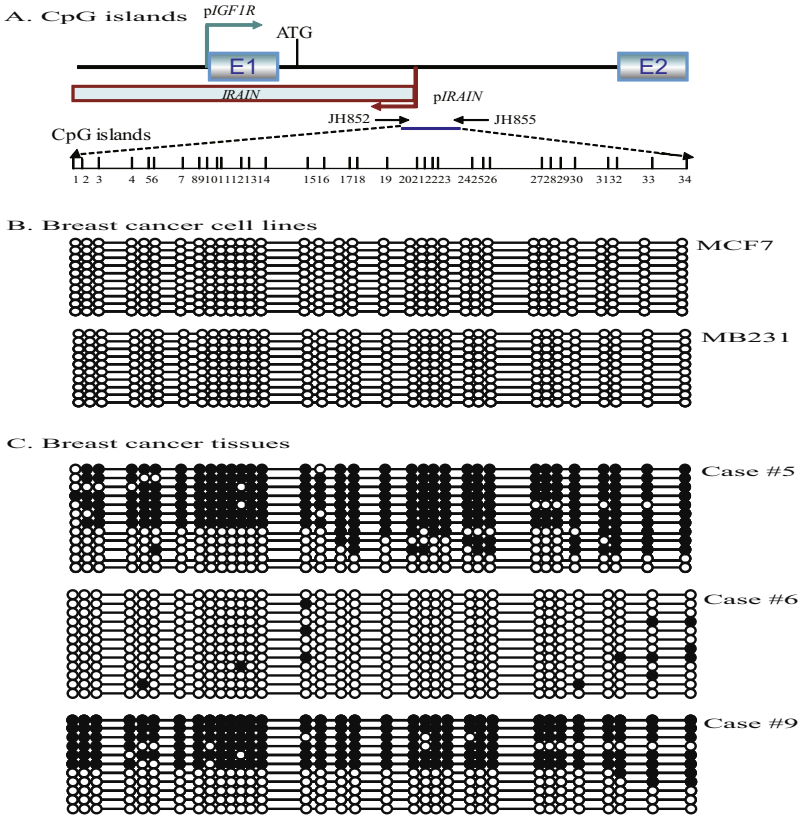

Fig. 4. DNA methylation in the regulation of the *IRF1R*/*IRAIN* locus. (A) Schematic diagram of CpG islands in the *IRAIN* promoter. Vertical lines: location of CpG islands. (B) DNA methylation of the *IRAIN* promoter in breast cancer cell lines (MCF7 and MDA-MB-231). Genomic DNAs were extracted from breast cancer cells. After treated with sodium sulphite, the *IRAIN* promoter DNA was amplified and sequenced. Open circles: unmethylated CpGs; solid circles: methylated CpGs. (C) CpG methylation of the *IRAIN* promoter in three breast cancer patients.

compared to peripheral blood leucocytes, breast cancer specimens had aberrant DNA methylation in the *IRAIN* promoter.

3.6. Aberrant imprinting of IRAIN lncRNA in breast cancers

Loss of *IGF2* imprinting is a very common epigenetic abnormality in cancer and may even be a prognostic biomarker [35]. In order to compare the status of *IRAIN* imprinting in peripheral blood leucocytes and breast cancer tissues, we studied five patients for whom both blood and surgery samples were available to track allelic expression.

As seen in Fig. 5A, all peripheral blood leucocyte cDNAs showed monoallelic expression of *IRAIN*. Four cases (#32, #36, #42, #37) expressed *IRAIN* lncRNA monoallelically from the 'A' allele, while case #39 expressed the 'G' allele. Surprisingly, in contrast to peripheral blood leucocytes, we found that in two cases (#36, #42), *IRAIN* expression switched to the 'G' allele in breast cancer specimens. In case #37, the breast cancer expressed the 'A' allele, while metastatic tumours switched to 'G' allele expression. These data suggest that in breast cancer tissues, *IRAIN* can undergo allelic switch, expressing the opposite allele as compared with that in circulating cells.

The *IRAIN* promoter was aberrantly unmethylated in breast cancer samples (Fig. 5B). Intriguingly, this aberrant demethylation pattern was also observed in peripheral blood leucocytes. Thus, it seems that breast cancer patients may undergo extensive alterations in promoter epigenotype. In the human *IGF2* gene, DNA demethylation is also a common epigenetic mutation observed in many human tumours [36–40].

4. Discussion

As the *IGF1R* signalling pathway is often aberrantly activated in tumours, including breast cancers, treatments using small molecule inhibitors and antibodies to block the tyrosine kinase activity have been advanced in preclinical and clinical testing [24]. In this communication, we have characterised *IRAIN*, a novel 5.4 kb intragenic non-coding RNA within the *IGF1R* locus in clinical samples collected from breast cancer patients. In cancer tissues, *IRAIN* is expressed in an antisense orientation within the *IGF1R* locus. A unique characteristic of this non-coding RNA is its monoallelic expression in breast cancers (Fig. 3A and B). By tracking the allelic expression in patient families, we demonstrate that *IRAIN* is transcribed from the paternal allele, while the copy from the maternal allele is silenced or imprinted (Fig. 3C), in agreement with the data in haematopoietic

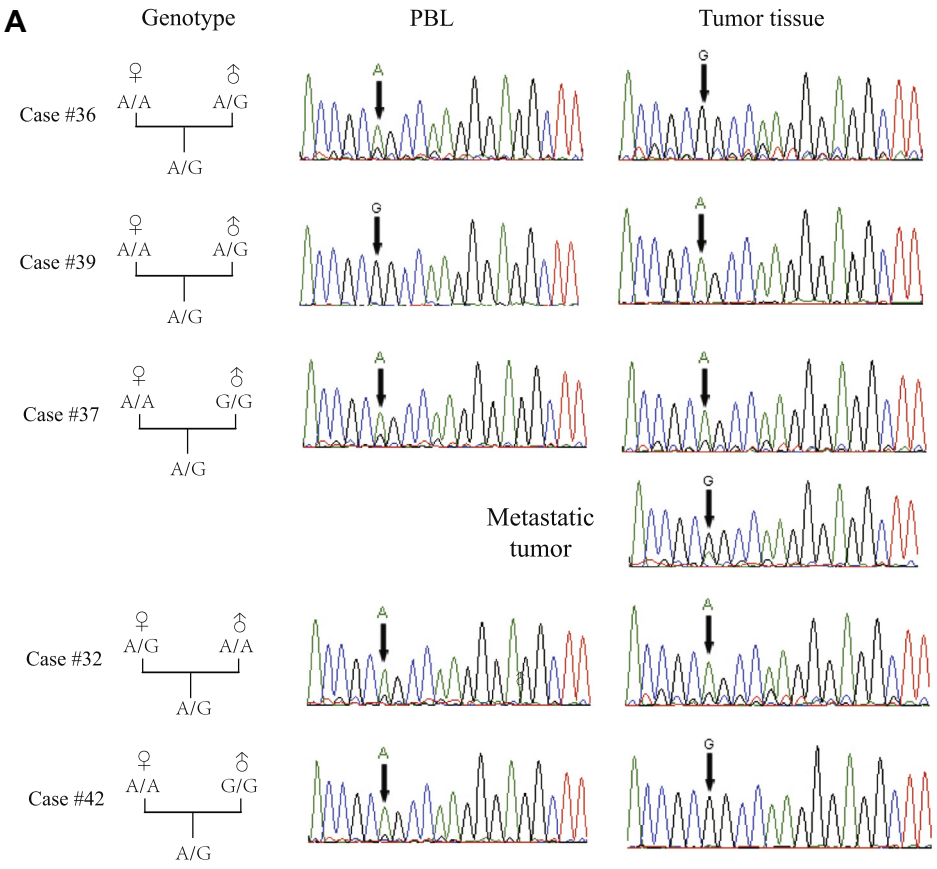

Fig. 5. Aberrant imprinting of IRAIN in breast cancer patients. (A) Allelic switch of *IRAIN* long non-coding RNA (lncRNA) imprinting. Allelic expression of *IRAIN* was examined by DNA sequencing of the single nucleotide polymorphism (SNP) rs8034564. Note the allele-switch of *IRAIN* lncRNA between the breast cancer tissues and peripheral blood leucocytes. PBL: peripheral blood leucocytes. (B) Aberrant DNA demethylation in the *IRAIN* promoter in allelic switching tumours. DNA methylation in CpG islands in the *IRAIN* promoter was quantitated by bisulphite sequencing. Open circles: unmethylated CpGs; solid circles: methylated CpGs. Note that in allelic switching tumours, there is extensive DNA demethylation of the *IRAIN* promoter in both breast cancer specimens and peripheral blood leucocytes.

malignancies [26]. Together, our data validate *IRAIN* as a new member of the family of imprinted genes [41].

By comparing the genotypes in informative tissues, it is interesting to note that *IRAIN* lncRNA seems to prefer the 'A' genotype expression (Table 1). In 18 heterozygous breast tissues examined, 89% expressed the 'A' allele. The 'G' allele, however, is rarely used by the host machinery for transcription. Similar cases have been reported in the *TMPRSS2* gene in prostate cancer stem cells [42]. In addition, stochastic monoallelic expression is also widespread in mammalian genomes, including olfactory receptor, *V1rb2* receptor, T-cell receptor and immunoglobulin genes, pheromone receptors, p120 catenin, odorant receptors, and protocadherins [43–47]. Allele-biased expression has also been observed in a number of putative schizophrenia (SZ) and autism spectrum disorder (ASD) SZ and ASD candidate genes, including *A2BP1* (*RBFOX1*), *ERBB4*, *NLGN4X*, *NRG1*, *NRG3*, *NRXN1*, and *NLGN1* [48]. Random monoallelic expression in the brain is related to epidemiological features of neuropsychiatric disorders [49,50]. In this study, however, it is still unclear if the preferential 'A' allele expression is associated with the function of this non-coding RNA.

Several genes undergo aberrant imprinting in cancers [51–53]. The most extensively studied example is the paternally-expressed *IGF2* gene. In many tumours, both parental copies of the *IGF2* gene may become fully expressed [54–56]. Reactivation of the normally-suppressed *IGF2* (imprinted) maternal allele, known as loss of imprinting (LOI), is a hallmark of many human tumours, especially childhood tumours [54–61] and cancer stem cells [62]. In this study, we did not observe biallelic expression of *IRAIN* in tumours. However, we did show an *IRAIN* epigenetic abnormality in breast tumour specimens. In normal tissues, *IRAIN* lncRNA is expressed from the maternal allele. However, in breast cancer tissues, the expression of *IRAIN* lncRNA switches to the alternative parental allele (Fig. 5A). The mechanisms underlying this allele-switch in breast cancer are not known. It is also unclear if this aberrant allelic switch will affect the activity of the *IGF1R* signal pathway in breast cancer. CRISPR Cas9 RNA genome editing has been recently used to study gene function [63,64], and it

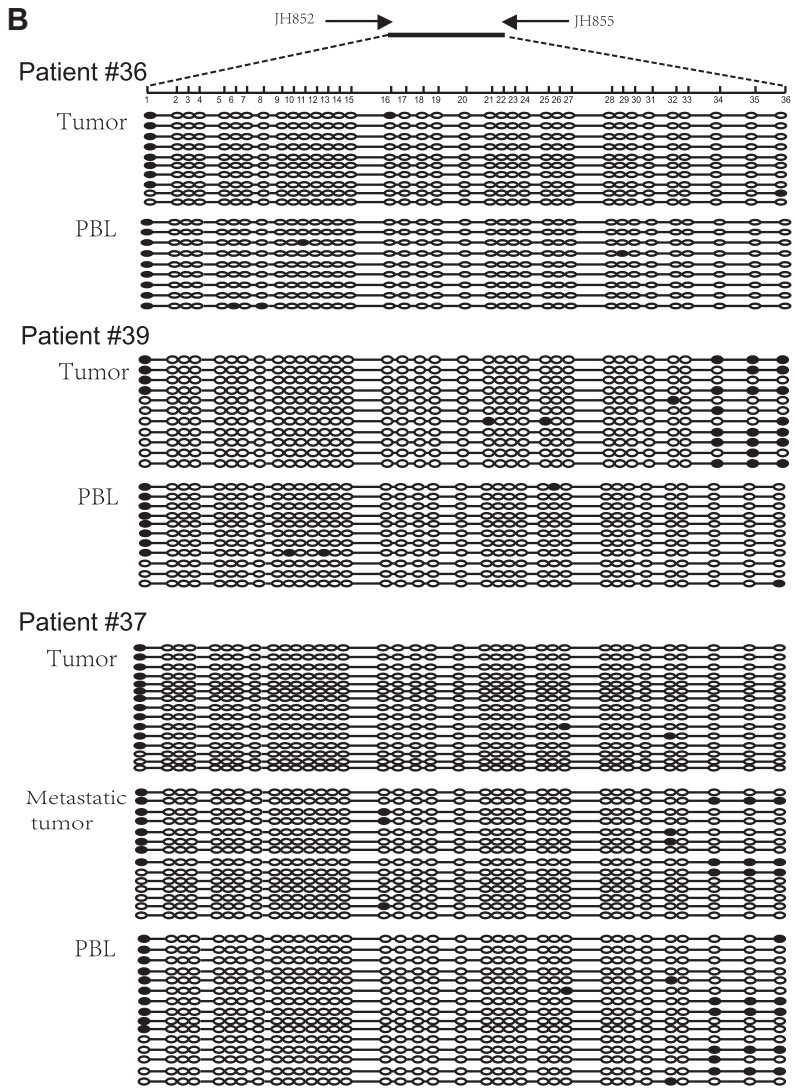

Fig 5. *(continued)*

would be interesting to learn if knockdown of *IRAIN* using this approach would affect *IGF1R* expression and thus the activity of the IGF signal pathway in tumours.

Allelic expression of sense and antisense RNAs is usually coupled via a *cis* transcription competition mechanism. A typical example is the mouse sense *Igf2r* coding RNA and the *Airn* antisense non-coding RNA, which are reciprocally imprinted [65–67] and tightly coordinated by DNA methylation in the *Airn* promoter [68]. The maternal *Airn* promoter is silenced by CpG island hypermethylation. Lack of the *Airn* lncRNA *cis*-competition leads to the expression of *Igf2r* from the maternal allele. In contrast, the unmethylated paternal *Airn* promoter leads to lncRNA expression, silencing the *Igf2r* promoter using a *cis* regulation mechanism [68,69]. In this study, however, we found that allelic expression between the *IRAIN* lncRNA and the *IGF1R* coding RNA is totally uncoupled. While the *IRAIN* lncRNA is monoallelically expressed (Fig. 3), the *IGF1R* coding mRNA is known to be biallelically expressed [26,70,71]. However, the fact that both *IRAIN* antisense lncRNA and *IGF1R* sense RNA are transcribed from the paternal chromosome without transcription competition or inhibition may provide a unique model to study imprinting mechanisms [66,67].

In summary, we have identified *IRAIN* as a novel maternally imprinted lncRNA located within the human *IGF1R* locus. In breast cancers, *IRAIN* undergoes aberrant allelic switching. However, many questions remain to be explored regarding this aberrant imprinting. For example, what is the impact of *IRAIN* expression/imprinting in the development of breast cancers? Could the aberrant allele-switch of *IRAIN* lncRNA be a prognostic biomarker? Is *IRAIN* lncRNA a predictive marker for *IGF1R* targeted therapies? Does the down-regulation of the *IRAIN* lncRNA in TNB and HER2+ samples correlate with clinic outcomes? Future studies are needed to address these questions. Detection of aberrant IGF2 imprinting in circulating leucocytes represents a valuable biomolecular marker for predicting individuals with high risk for colorectal cancer [38]. It would be interesting to learn whether the aberrant

allele-switch of *IRAIN* lncRNA can be utilised as a prognostic biomarker to assess breast cancer risk.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2014.10.031.

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