Directing HER4 mRNA expression towards the CYT2 isoform by antisense oligonucleotide decreases growth of breast cancer cells \textit{in vitro} and \textit{in vivo}

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\textbf{Background:} The tyrosine kinase receptor HER4 is a member of the epidermal growth factor receptor (EGFR) family. It plays diverse roles in cancer development and cancer progression and can both exert oncogenic and tumour-suppressive activities. Alternatively spliced isoforms of HER4 are critical to the different signalling possibilities of HER4.

\textbf{Methods:} We use a splice-switching oligonucleotide (SSO) to direct the alternative splicing of HER4 from the CYT1 to the CYT2 isoform in HER4-expressing breast cancer cells.

\textbf{Results:} Treatment with a target-specific SSO was accompanied by a decreased growth of the cells \((P<0.0001)\). In addition, the SSO treatment induced a decreased activity of Akt. We confirmed the SSO-dependent switching of the HER4 isoform CYT1 to CYT2 expression in a xenografted mouse tumour model driven by subcutaneously injected MCF7 cells. We hence demonstrated the feasibility of SSO-directed splice-switching activity \textit{in vivo}. Furthermore, the SSO treatment efficiently decreased the growth of the xenografted tumour \((P = 0.0014)\).

\textbf{Conclusion:} An SSO directing the splicing of HER4 towards the CYT2 isoform has an inhibitory effect of cancer cell growth \textit{in vitro} and \textit{in vivo}. These results may pave the way for the development of new anticancer drugs in HER4-deregulated cancers in humans.
and HER4 expression is often absent in hereditary breast cancer (Aqeilan et al, 2007; Zeng et al, 2008). In contrast, the CYT1 variant but not the CYT2 variant was associated with poor survival (Paatero et al, 2013). These data suggest that CYT1 is the more tumourigenic cytoplasmic isoform of HER4.

An evaluation of the potential of HER4 as a target for cancer therapy should take into consideration whether it is desirable to target the total pool of HER4 isoforms or only specific isoforms. An antibody specific for the JM-a isoform of HER4 has been found to inhibit the growth of breast cancer cells (Hollmen et al, 2009); however, apart from this, no studies have explored the effect of inhibiting specific splicing isoforms of HER4 on cancer cell growth.

Exon skipping to favour the expression of one splice variant over another can be done with splice-switching oligonucleotides (SSOs) (reviewed by Sazani and Kole, 2003). By hybridisation to pre-mRNA, SSOs sterically block sequences important for alternative splicing. This prevents exon recognition by the splicing machinery and causes the exon to be skipped (Sierakowska et al, 1996). Modifications to the oligonucleotide backbone such as 2′-O-methyl, 2′-O-methoxethyl, and locked nucleic acids (LNAs) prevent SSO-induced antisense downregulation via RNaseH and also stabilise the oligos towards nucleolytic degradation. These modifications also usually increase the binding affinity of the pre-mRNA target, and the increased competitiveness with spliceosome may account for the higher efficacy in vivo (Sazani and Kole, 2003; Roberts et al, 2006). The splice-switching capacities of SSOs have been demonstrated in several cell culture studies (Cartegni and Krainer, 2003; Scaffidi and Misteli, 2005; Williams and Kole, 2006; Khoo et al, 2007). More recently, successful SSO-directed splice-switching activity in mouse models has opened up the possibility that SSOs can have therapeutic applications in human diseases in which modulation of alternative splicing is desirable. Most advanced are the clinical trials on patients suffering from Duchenne muscular dystrophy, where SSO-mediated restoration of the aberrant splicing of the dystrophin pre-mRNA has shown some efficacy (van Deutekom et al, 2007; Graziewicz et al, 2008; Svasti et al, 2009; Bauman et al, 2010).

The purpose of our study was to investigate the possibility of targeting only one cytoplasmic isoform of HER4 and to further study the different functions of CYT1 and CYT2. We have investigated the ability of an SSO molecule to direct HER4 mRNA splicing towards the CYT2 isoform at the expense of the CYT1 isoform and demonstrate that a particular SSO can effectively decrease the CYT1/CYT2 ratio. The alteration of HER4 isoform expression is accompanied by a decreased growth of breast cancer cells. This is further demonstrated in a xenograft mouse model in which the SSO-induced switch in alternative splicing towards the CYT2 isoform of HER4 in the inoculated tumour causes decreased tumour growth.

MATERIALS AND METHODS

Cell lines. The human breast cancer cell lines MCF7 and T47D were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were tested for growth properties, morphology, mycoplasma contamination, and species by ATCC. Cells were cultured at 37°C in a humidified air atmosphere with 5% CO₂. The MCF7 cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and T47D cells were grown in RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% hepes, and 1% pyruvate (all media and supplements were from Life Technologies Carlsbad, CA, USA).

Oligonucleotide treatment. A 15-mer-LNA modified oligoribonucleotide SSOe26 (5′-l-T(mU)l(A)m(C)m(U)l(JC)m(C)m(U)l(JG)m(a)m(C)l(A)m(U)l(JG)m-3′ (l=LNA, m=methylated)) antisense to the 5′ splice site of HER4 exon 26 was
used. A scrambled oligonucleotide, SSOsc, containing the same nucleotides as SSOe26 at scrambled positions and modifications at the same positions as in SSOe26, was used as a negative control (5'-lG (m)T (b)T (b)T (m)C (m)A (U)G (m)T (m)C (b)G (m)C (m)A (T)T (m)A (b)C (b)T (b)G-3'). Both oligonucleotides were synthesised and purified by RiboTask ApS, Langeskov, Denmark.

Cells were plated in serum-free medium 24 h before oligonucleotide treatment. The cells were treated with oligonucleotides complexed with Lipofectamine 2000 (Life Technologies) according to the manufacturer’s directions at the concentrations indicated in the figures. Analyses were carried out 24 h after transfection for quantitative reverse transcription PCR (Q-PCR) and western blotting or continuously for cell proliferation assays.

RNA isolation and Q-PCR. The RNA was purified according to the manufacturer’s directions (Qiagen ‘Total RNA isolation kit’ (Qiagen, Chatsworth, CA, USA)) and resuspended in RNase-free water. The amount of purified RNA was quantified by UV spectroscopy (A260 nm = 1 corresponds to 40 μg ml⁻¹ RNA). Complementary DNA (cDNA) synthesis for the total HER4 assay was obtained from 0.1 μg of RNA and 0.4 μg of RNA was used for cDNA synthesis for the CYT1 and CYT2 assays. For the reverse transcription (RT) reaction was used: 1 × PCR buffer (Life Technologies, Carlsbad, CA, USA), 63.3 nmol⁻¹ MgCl₂ (Applied Biosystems), 25 nmol⁻¹ each of dATP/dTTP/dGTP and dCTP (Pharmacia), 2.5 mmol⁻¹ 16-mer oligo dt primer (DNA Technology), 20 U RNase inhibitor (Life Technologies), and 50 U reverse transcriptase (Life Technologies). Temperature profile was as follows: 50 °C for 30 min; 95 °C for 5 min; 4 °C end.

cDNA (1 μl) was used for Q-PCR using SYBR green Master (Roche, Basel, Switzerland) on a LightCycler 480 at: 95 °C for 10 min; 50 cycles of 95 °C for 10 s, specific annealing temperature for 10 s, 72 °C for 5 s; 99 °C for 1 s; 59 °C for 15 s; 95 °C for 1 s; and cooling to 40 °C. The forward and reverse primers were: HER4: forward: 5'-ACACGAGCTACGAGCTTTGGGCG-3'; reverse: 5'-GCCACTACGAGCTTTGGGCG-3'. CYT1: forward: 5'-GGATGAAGAGATTGGAAG-3'; reverse: 5'-TCCTGACATGGGGTTGTTGTA-3'. CYT2: forward: 5'-GAATAGGAACCAGTTTGTATCTCGG-3'; reverse: 5'-ACAGCAGGATCATCGAAAATC-3'. Annealing temperatures were: HER4: 64 °C; CYT1 and CYT2: 56 °C.

Western blotting. Cells were collected in scraping buffer and pelleted by centrifugation. Cell pellets were incubated on ice for 20 min with 1 ml of cell suspension was subsequently added (5000 cells per well). Cells were incubated for 30 min at room temperature before placing the E-plate in the Real-Time Cell Analyzer (RTCA) station. Impedance was measured every 1 h. At 24 h post cell seeding, 50 μl of media containing Lipofectamine 2000 or oligonucleotides complexed with Lipofectamine 2000 was added. Cell index was measured using the software provided by the manufacturer and normalised to the time just after treatment.

Tumour xenografts. At 4 days before cell inoculation, a β-estradiol pellet (0.5 mg, 60-day release; Innovative Research of America, Sarasota, FL, USA) was implanted subcutaneously in the neck region of the mouse to support tumour growth. MCF7 cells (4 × 10⁶ cells in 200 μl of PBS) were injected subcutaneously into the right flanks of each mouse (9 weeks old, female, nu/nu BALB/c (C.Cg/A BamB/c Taconic, Foxn1nu, Taconic, Ejby, Denmark)). Isoflurane (Abbott, Copenhagen, Denmark) was used for anaesthesia for implantation of pellets and inoculation of tumour cells. Tumours were allowed to grow until visible (1 week; average volume 83 mm³ (tumour volume = ½ (length × width²)) before they were randomly allocated into two groups with approximately the same total tumour volume in each group (6 mice in each group). Treatment was performed with 400 μg SSOe26 or SSOsc in 150 μl PBS intraperitoneally three times per week. Tumour sizes were measured at the days of treatment by caliper measurements of the largest longitudinal (length) and transverse (width) diameter; the tumour volume was then calculated by the formula given above. At the end of the experiment, the mice were killed and the tumours were removed and snap-frozen for RNA isolation. The tumours were mechanically homogenised and RNA was extracted as described above. Animal experiments were conducted with approval from The Danish Experimental Animal Inspectorate.

Statistics. Differences in mRNA expressions were analysed using t-tests.

Cell growth and in vivo tumour growth were analysed using univariate ANOVA models for repeated measurements on the log-transformed data.

RESULTS

SSOe26 shifts the splicing equilibrium of the CYT1 and CYT2 isoforms of HER4. A 15-mer LNA-modified oligonucleobonucleotide, SSOe26 (SSO exon 26; Figure 1A), was designed to anneal to the 5’ splice site of exon 26 of the HER4 pre-mRNA (Figure 1B). The nucleotide sequence of SSOe26 was scrambled to create a control oligo, SSOsc (SSO scrambled). By annealing to the 5’ splice site of exon 26, SSOe26 makes the splice site inaccessible for the splicing machinery, which results in exon skipping and thereby expression of the CYT2 isoform (Figure 1C).

Both MCF7 and T47D, two breast cancer cell lines, express HER4. To determine the relative CYT1 and CYT2 expression in these two cell lines, a semiquantitative PCR assay was performed. For this assay one set of primers anneal to either site of exon 26 to give PCR products of different size depending on splice variant expression. The isoforms can be visualised upon gel electrophoresis. This assay revealed that in both cell lines, the CYT1 variant is the predominant cytoplasmic isoform of HER4 (Figure 2A). The MCF7 and T47D cells were then transfected with SSOe26 at increasing concentrations as indicated in Figures 2B and C. Cells were harvested 24 h after transfection, and CYT1 and CYT2 mRNA expression was quantified by Q-PCR. A dose-dependent decrease in the ratio of the CYT1/CYT2 isoform expression was induced by SSOe26 treatment (Figures 2B and C). For the MCF7 cells, a
Figure 1. Sequence and annealing position of SSOe26. (A) Sequence and backbone modifications of SSOe26. (I) = LNA, (m) = 2′-O-methyl. (B) Part of the HER4 pre-mRNA sequence showing the 3′-end of intron 25, exon 26 (italic) and the 5′-end of intron 26. The sequence of 15 nucleotides annealing with SSOe26 is underlined. The numbers indicate the nucleotide number of the first and the last nucleotide in exon 26 according to NM_005235.2, NCBI. (C) Alternative splicing of exon 26 in the HER4 pre-mRNA. Inclusion of exon 26 in the HER4 pre-mRNA generates the CYT1 isoform. Skipping of exon 26 generates the CYT2 isoform.

Figure 2. The effect of SSOe26 and SSOSc on HER4 CYT1/CYT2 mRNA ratio and on total HER4 mRNA and protein expression. (A) Agarose gel electrophoresis depicting the mRNA expression of the CYT1 and CYT2 isoforms in MCF7 and T47D cells. The CYT1 amplicon contains additional 45 amino acids compared with CYT2 and, hence, CYT1 is more retarded in the gel. Bands representing CYT1 and CYT2 are indicated in the figure. A third of ~300 bp represents an artefact as a result of the smaller products annealing together (Elenius et al., 1999). (B) The mRNA expression ratio of CYT1 and CYT2 in MCF7 cells treated with increasing concentrations of SSOe26. The cells were treated with fresh media containing transfection reagent (control) or indicated amounts of SSOe26 or SSOSc formulated with transfection reagent as indicated. Bars represent means of four individual samples, error bars are s.e.m. Asterisks indicate the level of significance (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; and ****P ≤ 0.0001). (C) The mRNA expression ratio of CYT1 and CYT2 in T47D cells treated with increasing concentrations of SSOe26. The cells were treated with fresh media containing transfection reagent (control) or indicated amounts of SSOe26 or SSOSc formulated with transfection reagent as indicated. Bars represent means of four individual samples, error bars are s.e.m. Asterisks indicate the level of significance (**P ≤ 0.01). (D) Total HER4 mRNA expression relative to the mRNA expression of the HMBS household gene. The cells were treated with fresh media containing transfection reagent (control) or 100 nm of SSOe26 or 100 nm of SSOSc formulated with transfection reagent as indicated. Bars represent means of four individual samples, error bars are s.e.m. (E) Total HER4 protein expression. The cells were treated with fresh media containing transfection (control) reagent or 100 nm of SSOe26 or 100 nm of SSOSc formulated with transfection reagent as indicated. Stripping and reblotting with β-actin demonstrates equal loading.

decrease was seen with as little as 1 nm of SSOe26 (P = 0.0158), and with 100 nm SSOe26, the CYT1/CYT2 ratio was reduced to approximately half of the ratio of untreated cells (mean ratio for control cells: 0.51 ± 0.027 (s.e.m.); mean ratio for SSOe26-treated cells: 0.22 ± 0.015 (s.e.m.)). For the T47D, 50 nm of SSOe26 was necessary to give a significant decrease in the CYT1/CYT2 ratio (P = 0.0005). Treatment with SSOSc at the highest dose used for SSOe26 (500 nm) did not change the CYT1/CYT2 ratio significantly (P = 0.3894 for MCF7 cells and P = 0.1135 for T47D cells).

To ensure that binding of SSOe26 did not cause degradation of the HER4 pre-mRNA, total HER4 mRNA expression was quantified by Q-PCR in MCF7 cells (Figure 2D). The SSOe26 did not decrease mRNA expression of HER4. On the contrary, a small, although not significant (P = 0.0654), increase was observed. The SSOSc had no effect on HER4 mRNA expression. Also, on protein level, neither SSOe26 nor SSOSc induced a change in the HER4 expression as demonstrated by western blotting (Figure 2E).

Together, these results demonstrate that SSOe26 successfully induces splice switching of HER4 to decrease the ratio of CYT1/CYT2 isoform expression without inhibiting total HER4 expression.
SSOe26 decreases breast cancer cell growth. The effect of an SSO-induced decrease in the CYT1/CYT2 isoform ratio was assessed by real-time measurements of MCF7 and T47D cell growth. Compared with the control cells, splice switching of HER4 with SSOe26 caused an almost complete growth arrest (P < 0.0001; Figures 3A and C). The SSOsc had a negligible effect on cell growth. These results from a real-time growth experiment were reproduced using a colorimetric method to investigate cell growth (MTS assay, results not shown).

The SSOe26-induced inhibition of cell growth was dose dependent and decreased with decreasing concentrations of SSOe26 (Figures 3B and D).

SSOe26 alters the activity of signalling proteins and induces apoptosis. Two of the main pathways activated by HER4 are the MAPK pathway and the PI3K/Akt pathway, the latter specific for CYT1. We investigated how splice switching with SSOe26 influences the activity of these pathways. Phosphorylation of Akt in response to SSOe26 treatment was examined by western blotting and resulted in decreased phosphorylation of Akt compared with cells treated with SSOsc (Figure 4A). The total amount of Akt protein was unchanged upon treatment (Figure 4A). The phosphorylation status and the total protein content of MAPK as a result of SSOe26 treatment were also examined by western blotting. We detected no change in either the phosphorylation status or the total protein expression of this protein (Figure 4B).

SSOe26 induces splice switching and decreases tumour growth in mouse xenografts. After observing a splice-switching activity of SSOe26 in cell culture studies, the ability of SSOe26 to induce splice switching in vivo was tested. The MCF7 breast cancer cells were planted subcutaneously onto the right flank of immunodeficient mice and SSOe26 or SSOsc was hereafter injected intraperitoneally three times per week. After 15 days of treatment, the tumours were removed and CYT1 and CYT2 mRNA expression was quantified by Q-PCR.

In accordance with the cell culture experiments, tumours from mice receiving SSOe26 had a substantially lower CYT1/CYT2 mRNA ratio than tumours from mice receiving the control oligo (P < 0.0001), which demonstrates the splice-switching activity of SSOe26 in vivo (Figure 5A).

The effect of SSOe26 on the growth of the tumour xenografts was assessed by manually measuring the tumour size three times per week. The tumours of SSOe26-treated mice showed a significantly slower growth than the tumours of SSOsc-treated mice (P = 0.0014; Figure 5B).

The in vivo results clearly demonstrate the ability of SSOe26 to reach the subcutaneous location of the xenograft tumour from the intraperitoneal cavity site of injection and to induce
Splice switching of HER4 mRNA in cancer cells

DISCUSSION

Divergent data exist on the role of HER4 in tumour growth and development. In some settings, HER4 exerts tumourigenic phenotype characteristics in accordance with the biology of its family members EGFR and HER2; however, in other settings, tumour HER4 expression is an indicator of a better survival of cancer patients (Hollmen and Elenius, 2010). The existence of alternatively spliced isoforms of the receptor most likely contributes to the contradictory data on HER4 because the alternatively spliced isoforms have been reported to exert different biological effects (Veikkolainen et al, 2011). The HER4 has been described as a possible new specific target for cancer therapy in numerous connections (Kurppa and Elenius, 2009; Settleman, 2009; Rudloff and Samuels, 2010). For this purpose, targeting only one isoform of HER4 may be preferable. We investigated the potential of using SSO to modulate the splicing of HER4 pre-mRNA into its CYT1 and CYT2 isoforms. The purpose of this was to investigate the possibility of targeting only one cytoplasmic isoform of HER4 and to further study the different functions of CYT1 and CYT2.

The use of an SSO that targets the junction of intron 26 and exon 26 resulted in a successful modulation of the HER4 pre-mRNA that decreased the CYT1/CYT2 ratio both in vitro and in vivo. The ability of the SSO to successfully change the expression of HER4 cytoplasmic isoforms makes SSO molecules a useful tool for investigating the function of the CYT1 and CYT2 splice variants. The majority of studies on differences between CYT1 and CYT2 functions rely on an exogenous expression of the full-length HER4 or the ICD of the HER4, which leads to excessive overexpression of the gene. In contrast, the SSO functions in settings where expression levels and background environments are physiologically relevant, which makes this an attractive alternative to overexpression.

One of the purposes of backbone modifications of the SSO is to prevent mRNA degradation by RNA interference. In our studies, the mRNA expression level was, indeed, conserved. On the contrary, we observed a slight increase in the total HER4 mRNA level. This increase could be a result of regulatory mechanisms responding to the binding to the mRNA and/or response to regulations of HER4 isoform expression patterns. The change in mRNA expression was not, however, translated to the protein level.

The SSO-induced decrease in the CYT1/CYT2 ratio was accompanied by a decrease in the growth of the HER4-expressing MCF7 and T47D breast cancer cells. This finding was supported by the ability of the SSO to inhibit tumour growth in the xenograft mouse model. It has previously been found that downregulation of endogenous HER4 in MCF7 breast cancer cells reduces cell growth in vitro and in vivo (Tang et al, 1999). Our results indicate that it is the downregulation of the CYT1 isoform of HER4 that gives rise to this growth inhibition. The CYT1, but not CYT2, directly couples to the PI3K/Akt pathway, and CYT1, but not CYT2, can execute an inhibitory effect on apoptosis via the PI3K/Akt pathway (Kainulainen et al, 2000). We did, indeed, see a decrease in Akt activity in cells upon switching from the CYT1 towards the CYT2 isoform. The downstream targets of Akt are numerous. In medulla blastoma cells, CYT1 overexpression has a PI3K/Akt-dependent inhibitory effect on apoptosis upon serum starvation or etopside treatment (Ferretti et al, 2006). Also, CYT1 has been found to induce chemotaxis (Kainulainen et al, 2000). In rat adrenal gland pheochromocytoma cells, CYT1 expression promoted less differentiation and increased cell proliferation compared with CYT2 (Veikkolainen et al, 2011). Together with our present results, these findings add to the picture of CYT1 as a more carcinogetic isoform than CYT2, and they suggest that targeting only this isoform of HER4 is therapeutically most relevant.

However, results have also been obtained that suggest that CYT1 and CYT2 have the opposite effects. In mammary epithelial cells, overexpression of the cleavable 80 kD cytoplasmic domain of CYT1 was found to decrease growth and to induce a more differentiated phenotype, whereas the cleavable 80 kD cytoplasmic domain of CYT2 increased growth and induced no differentiation (Muraoka-Cook et al, 2009). In addition, HER4 nuclear localisation is associated with poor survival and CYT2 has been found to enter the nucleus more readily than CYT1 (Junttila et al, 2005; Sundvall et al, 2007; Zeng et al, 2007).

The contradictory results regarding the roles of CYT1 and CYT2 demonstrate that the divergent roles of HER4 do not come down to cytoplasmic isoform expression alone. Besides isoform expression, HER4 functions are influenced by the activating ligand, the dimerisation partner, and the localisation of cleavage products. That the activating ligand is important also in regard to HER4 cytoplasmic isoform expression was demonstrated in Madin-Darby Canine Kidney (MDCK) II cells in which HB-EGF had a greater proliferative effect than NRG1β in CYT2-expressing cells, whereas NRG1β had a greater proliferative effect than HB-EGF in CYT1-expressing cells (Zeng et al, 2007). With regard to the importance of the dimerisation partner, it has recently been found that HER2 expression is important for HER4 to possess an oncogenic phenotype (Mill et al, 2011b). In addition, a constitutively dimerised variant of HER4 possessed a proliferative potential.
different from that of ligand activation of a wild-type receptor in prostate cancer cell lines (Mill et al., 2011a).

This study is the first to selectively target only one cytoplasmic splice variant of HER4. Despite the contradictory results on CYT1 and CYT2 functions, there is no doubt that the cytoplasmic isoforms play a role in the divergent roles of HER4 and that in some cases, it will be attractive to target only one of these isoforms. Targeting only one isoform will potentially result in fewer side effects as other isoforms of HER4 can still exert their functions.

Example of conditions in which targeting of CYT1 could be attractive is medulla blastoma where the CYT1/CYT2 ratio was found to be higher in aggressive than in less aggressive tumours and in ovarian cancer in which the CYT1 but not the CYT2 isoform was associated with poor survival (Ferretti et al., 2006). Under the conditions of our experiments, we also found that the decrease in the CYT1/CYT2 ratio inhibited the growth of MCF7 breast cancer cells and mice in xenografted tumours. If this effect can also be achieved in humans, SSO targeted to HER4 may represent a novel strategy in cancer treatment in the future.

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2297
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