Mutational analysis of the insulin-like growth factor 1 receptor tyrosine kinase domain in non-small cell lung cancer patients

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Received March 17, 2015; Accepted April 2, 2015

DOI: 10.3892/mco.2015.580

Abstract. The insulin-like growth factor 1 receptor (IGF1R) pathway plays an important role in the pathogenesis of non-small cell lung cancer (NSCLC) and also provides a mechanism of resistance to targeted therapies. IGF1R is therefore an ideal therapeutic target and several inhibitors have entered clinical trials. However, thus far the response to these inhibitors has been poor, highlighting the importance of predictive biomarkers to identify patient cohorts who will benefit from these targeted agents. It is well-documented that mutations and/or deletions in the epidermal growth factor receptor (*EGFR*) tyrosine kinase (TK) domain predict sensitivity of NSCLC patients to EGFR TK inhibitors. Single-nucleotide polymorphisms (SNPs) in the IGF pathway have been associated with disease, including breast and prostate cancer. The aim of the present study was to elucidate whether the *IGF1R* TK domain harbours SNPs, somatic mutations or deletions in NSCLC patients and correlates the mutation status to patient clinicopathological data and prognosis. Initially 100 NSCLC patients were screened for mutations/deletions in the *IGF1R* TK domain (exons 16-21) by sequencing analysis. Following the identification of SNP rs2229765, a further 98 NSCLC patients and 866 healthy disease-free control patients were genotyped using an SNP assay. The synonymous SNP (rs2229765) was the only aberrant base change identified in the *IGF1R* TK domain of 100 NSCLC patients initially analysed. SNP rs2229765 was detected in exon 16 and was found to have no significant association between IGF1R expression and survival. The GA genotype was identified in 53.5 and 49.4% of NSCLC patients and control individuals, respectively. No significant difference was found in the genotype (P=0.5487) or allele (P=0.9082) frequencies between the case and control group. The present findings indicate that in contrast to the *EGFR* TK domain, the *IGF1R* TK domain is not frequently mutated in NSCLC patients. The synonymous SNP (rs2229765) had no significant association between IGF1R expression and survival in the cohort of NSCLC patients.

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Key words: insulin-like growth factor 1 receptor, single-nucleotide polymorphism, non-small cell lung cancer, targeted therapy

Introduction

The role of the insulin-like growth factor 1 receptor (IGF1R) in the pathogenesis of malignant epithelial tumours, including non-small cell lung cancer (NSCLC), has been well-characterised (1-5). Activation of this receptor pathway promotes tumour growth by inhibition of apoptosis, transformation, metastasis and induction of angiogenesis through vascular endothelial growth factor (6-10).

IGF1R is frequently overexpressed in NSCLC patients, however, there is controversy over its significance as a prognostic marker. Certain studies have shown no correlation between high IGF1R expression and patient survival (3,4); conversely, other studies have demonstrated that high IGF1R expression was associated with nodal metastasis, recurrence and a significantly poorer overall survival (OS) rate in NSCLC patients (11,12). A recent meta-analysis suggests IGF1R positive expression as an adverse factor for disease-free survival (DFS) in NSCLC patients and reports the correlation to smoking status and tumour size, but there was no significant association between IGF1R expression and OS on univariate or multivariate analysis (13). Ludovini *et al* (14) reported that high co-expression of IGF1R and epidermal growth factor receptor (EGFR) is associated with a shorter DFS in resected NSCLC patients and a trend towards a poorer OS. We have previously shown that high co-expression of EGFR and IGF1R correlates with poor patient prognosis in resected NSCLC (15).

Following the success of other targeted therapies, such as the EGFR inhibitors, IGF1R also emerged as an attractive therapeutic target. Although the results from early clinical trials targeting IGF1R showed certain promise, larger randomized phase III trials have not shown a clear clinical benefit of

targeting this pathway in combination with chemotherapy (16). The results of these trials and others involving targeted agents have demonstrated the importance of identifying predictive biomarkers to select the appropriate patient population who will benefit from treatment.

Somatic mutations in the kinase domain of a receptor can cause the cell to become highly dependent on the constitutively active receptor signalling pathway. These aberrations can also cause conformational changes that can impact on the binding capabilities of a therapeutic agent targeting this region (17-22). As many as 2,412 single-nucleotide polymorphisms (SNPs) have been identified in *IGF1R* and several have been associated with a cancer risk (22-26). A common polymorphism of the *IGFIR* gene (G1013A) has been shown to modify the risk of obesity for esophageal adenocarcinoma and, in combination with a polymorphism in *IGF2R* (G1619A), is an independent prognostic factor in advanced NSCLC (27-29). A previous study has shown that the IGF1 pathway polymorphisms are potential predictive/prognostic molecular markers for cetuximab efficacy in wild-type *KRAS* colorectal cancer patients (30). These polymorphisms may activate crosstalk between the IGF1R and EGFR signalling pathways. A study by Deming *et al* (31) on genetic variation in patients with breast cancer found that SNP rs951715 within the *IGF1R* gene was associated with breast cancer survival in postmenopausal women. Another polymorphism, SNP rs2229765, appears to be a silent mutation with no correlation to survival rate in breast cancer patients and thus far has no association with any epidemiological traits. In a retrospective study of 304 NSCLC patients who underwent curative pulmonary resection, 1 silent mutation in exon 16 and 3 intronic mutations were detected within the *IGF1R* gene but did not correlate to IGF1R protein expression (32).

Identifying functional polymorphisms in the IGF1R pathway could be used to select patients who may benefit from IGF1R-targeted agents. Thus far, there have been few reports of SNPs or somatic mutations in the *IGF1R* tyrosine kinase (TK) domain. Therefore, the aim of the present study was to screen NSCLC patients, who had undergone lung tumour resection surgery, for gene aberrations in the *IGF1R* TK domain.

Materials and methods

Subjects. This is a retrospective study in which a database of all the patients who underwent curative-intent surgical resection of a primary tumour at St. James's Hospital, Dublin (Republic of Ireland) between February 2001 and February 2005, was analysed. A cohort of 198 stage I-III NSCLC patients, staged according to the International System of Staging for Lung Cancer (33), was randomly selected from the database. Information on baseline demographics, clinicopathological characteristics and surgical approach was collected following a review of clinical notes and histopathology reports. Outcome data, including peri-operative mortality and long-term survival, were updated prospectively. Patient characteristics are detailed in Table I. The study was approved by the St. James's Hospital Ethics Committee. Controls (n=866) were ascertained with written informed consent from the Trinity Biobank and represented blood donors from the Irish Blood Transfusion Service recruited in the Republic of Ireland. Individuals taking regular prescribed medication are excluded from blood donation in the Republic of Ireland and donors are not financially remunerated.

DNA extraction. DNA was extracted from 3x10-μm formalin-fixed paraffin-embedded sections from NSCLC patients and prepared using the QIAamp® DNA kit. DNA was extracted from control blood samples using the Gentra Autopure system (Qiagen, Hilden, Germany).

PCR amplification of exons 16-20. The primer sequences used for the PCR reactions are outlined in Table II. The following PCR conditions were used: 1X GoTaq® Green Master mix (Promega Corp., Madison, WI, USA), 5 μM of forward and reverse primers and 100 ng of DNA made up to a volume of 50 μl with dH_20. A pre-PCR heat step of 94˚C for 5 min was carried out to activate the enzyme and the DNA was amplified for 35 cycles at 94˚C (1 min), 56˚C (1 min) and 72˚C (1 min), and at 72˚C (10 min) after the last cycle. A portion of the PCR product was electrophoresed on a 1.4% agarose gel to verify product integrity. PCR products were purified using the Qiagen QIAquick PCR purification kit. The DNA was measured using the NanoDrop 1000 spectrophotometer.

Sanger sequencing of PCR products. Sanger sequencing reactions were set up as follows: 2 μl BigDye® Terminator mix v3.1, 50 ng DNA, 5 μM forward or reverse primers and 2 μl sequencing buffer, diluted to 20 μl with water. A positive control was also set up to ensure the efficiency of the sequencing reaction (1 μl pGem, 2 μl M13 primer, 2 μl BigDye® Terminator mix v3.1 and 2 μl sequencing buffer). The pGem and BigDye® Terminator mix v3.1 were sourced from Applied Biosystems (Warrington, UK). Sequencing was performed on a 3130xl genetic analyser (Applied Biosystems, Foster City, CA, USA) and sequencing files were analysed using the BioEdit v 7.0.8 (Ibis Biosciences, Carlsbad, CA, USA).

SNP genotyping assay. A TaqMan® SNP genotyping assay (Applied Biosystems) that detected the SNP at codon 3179A>G (rs2229765) in exon 16 of the TK domain by quantitive PCR was also used to screen patients. TaqMan® SNP Genotyping Assay 5' nuclease technology uses two allele-specific TaqMan® MGB probes and a PCR primer pair to detect the specific SNP target. The probes and primers uniquely align with the genome, enabling TaqMan® genotyping technology to provide unmatched specificity. Genotype data for rs2229765 was generated on the control samples using the Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc., Santa Clara, CA, USA) (34).

Immunohistochemistry. IGF1R immunohistochemical analysis was performed on 4-μm slides cut from 22 tissue microarrays and mounted on Superfrost Plus glass slides (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The entire staining procedure was performed on an automated immunohistochemistry device [BenchMark XT; Ventana Medical Systems, Inc. (VMS), Tucson, AZ, USA] following the manufacturer's instructions. In brief, slides were deparaffinized on the thermopads using the Ez Prep reagent (950-102; VMS). For epitope recovery, the standard CC1 buffer, a citric-acid-based antigen retrieval solution, was used (950-102; VMS). All the

Table I. Patient characteristics.

Characteristics	Cases, n	Controls, n
Total	198	866
Gender		
Male	125	296
Female	73	570
Smoking history		
Former	130	ND
Current	57	ND
Never	11	ND
Histology		
SCC	94	ND
ADC	84	ND
Other	20	ND
Age, years		
<50	28	698
>50	170	168

ND, not determined.

Table II. Primer sequences used for amplification of *IGF1R* RTK 1-6.

Exon	Primer sequence (5'-3')
RTK1	F: GGCTTGTTTCTGTACCTGCT
	R: AGCCAAGAACATACTGGGAG
RTK2	F: ACAACACAGGCATCAGCAAG
	R: GACACAGCATTTCCTTGCAG
RTK3	F: CTCGAAAGAAATTGGCATGG
	R: TCTCCAGGGGCAGACTAATG
RTK4	F: CTGCTCCAGCGTGTGACTCT
	R:GAGCTAAAGCTGGCAACGGG
RTK5	F: CTGCTCGGGATGTAAGAAGT
	R: CTCCTAATCTCCTGTGACCC
RTK6	F: CGTACGAGGTAAACAGGAG
	R: AGCTTGTTCTCCTCGCTGTA

IGF1R, insulin-like growth factor 1 receptor; RTK, receptor tyrosine kinase; F, forward; R, reverse.

subsequent washing and blocking steps followed standard protocols. Slides were incubated with the prediluted primary antibody (monoclonal rabbit anti-IGF1R, clone G11; VMS) for 16 min at 37˚C. Negative controls included identically processed slides in which the primary antibody was replaced by accordingly diluted non-immune rabbit IgG (Abcam, Cambridge, UK; 27478). Positive controls included identically treated paraffin slides of a H322M xenograft, an IGF1R-overexpressing NSCLC cell line. Detection of primary antibody binding was performed using the ultraView Universal DAB Detection kit (760-091; VMS). After the diaminobenzidine (DAB) reaction was developed, slides were counterstained with haematoxylin II (760-500; VMS), dehydrated in a serial dilution of ethanol, transferred in xylene and mounted with Eukitt (O. Kindler GmbH, Freiburg im Breisgau, Germany).

Table III. Characteristics for the SNP rs2229765, evaluated in the present study.

Characteristics	SNP
Gene name	*IGF1R*
Alleles, major>minor	G>A
SNP reference ID	rs2229765
Position in gene	Exon 16
Codon	Glu1013Glu

Polymorphism data on SNP rs2229765 was retrieved from the NCBI SNP reference database. SNP, single-nucleotide polymorphism.

Table IV. Genotypic and allelic frequency of SNP rs2229765 in NSCLC and control patients.

Frequency	NSCLC patients (n=198), n (%)	Control patients (n=866), n (%)	P-value
Genotype			
AA	35 (17.7)	175 (20.2)	0.5487
GA	106 (53.5)	428 (49.4)	
GG	57 (28.8)	263 (30.3)	
Allele			
A	176 (44.4)	778 (44.9)	0.9082
G	220 (55.5)	954 (55.0)	

SNP, single-nucleotide polymorphisms; NSCLC, non-small cell lung cancer.

Statistical analysis. The software package SPSS v16.0 (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis. χ^2 test, Cox regression analysis, Kaplan-Meier analysis and the log-rank test were used to illustrate the significance of various clinical characteristics. Assumption of the proportional hazard was tested for all covariates. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Initially, 100 NSCLC patients were screened for the presence of mutations, deletions or SNPs in exons 16-20 of the TK domain of *IGF1R*. The polymorphism (rs2229765) located on exon 16 of the *IGF1R* gene (GenBank accession NM_000875), consisting of a G to A transition at nucleotide 3174 but not leading to an amino acid change (Glu->Glu) at position 1043 (E1043E) (GenBank accession NP_000876), was identified (Table III). No other mutations, deletions or SNPs were found in the TK domain. A further 98 NSCLC patients were screened for the presence of the same SNP (rs2229765) and compared to the control disease-free individuals (n=866). The control patient DNA used came from

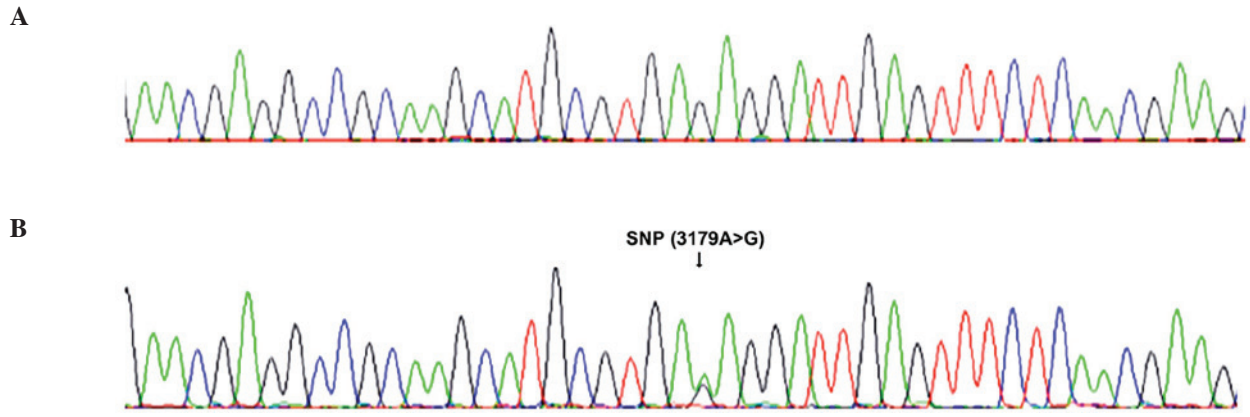

Figure 1. Forward sequence traces from a non-small cell lung cancer (NSCLC) patient with (A) no single-nucleotide polymorphism (SNP) (homozygous G/G) and (B) a heterozygous SNP (SNP 3179A>G) in exon 16.

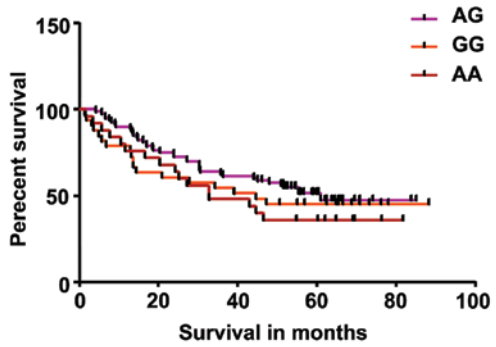

Figure 2. Kaplan-Meier plot of genotype distribution of SNP rs2229765 in non-small cell lung cancer (NSCLC) patients (log rank test P=0.3564).

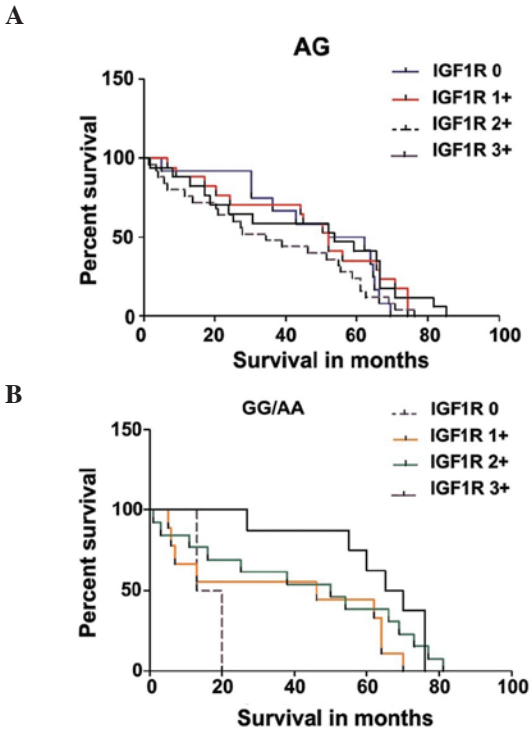

Figure 3. Kaplan-Meier plot correlating insulin-like growth factor 1 receptor (*IGF1R*) expression to genotype distribution of SNP rs2229765 in non-small cell lung cancer (NSCLC) patients. (A) Heterozygous A/G, (B) homozygous dominant GG and homozygous recessive AA. Log rank test (A) P=0.3005 and (B) P=0.1164.

the Trinity Biobank in the Institute of Molecular Medicine, Trinity College Dublin. These results where subsequently correlated to patient survival/pathological data. Example sequence traces from a homozygous G/G patient and a heterozygous A/G patient are shown in Fig. 1A and B, respectively.

The heterozygous GA genotype was found in 53.5 and 49.4% of NSCLC patients and controls, respectively (Table IV). The dominant GG genotype was identified in 28.8 and 30.3% of NSCLC patients and control individuals, respectively, while the recessive AA genotype was found in 17.7 and 20.2% of NSCLC patients and control individuals, respectively. No significant difference was found in the genotype (P=0.5487) frequency between cases and controls. The A allele was identified in 44.4 and 44.9% of NSCLC patients and control individuals, respectively, while the G allele was identified in 55.5 and 55% of NSCLC patients and control individuals, respectively. No significant difference was identified in the allelic (P=0.9082) frequency.

From the overall patient and control cohorts, patients >70 years were excluded as there were no matched controls available for this age group. Age and gender matching ensures that any difference between cases and controls is disease-related. The genotypic and allelic frequencies were subsequently examined in 95 NSCLC patients and 95 age- and gender-matched control individuals. When patients were age- and gender-matched the GA genotype was identified in 56.8 and 48.4% of NSCLC and control patients, respectively (Table V). The dominant GG genotype was identified in 22.1 and 29.4% of NSCLC patients and control individuals, respectively, while the recessive AA genotype was identified in 21.0 and 22.1% of NSCLC patients and control individuals, respectively. No significant difference was identified in the genotype (P=0.4351) frequency. The A allele was identified in 49.4 and 46.3% of NSCLC patients and control individuals, respectively, while the G allele was identified in 50.5 and 53.6% of NSCLC patients and control individuals, respectively. No significant difference was found in the allelic (P=0.2636) frequency.

IGF1R expression and survival data were available for 100 NSCLC patients. IGF1R expression was compared to the results of the genotype distribution in NSCLC patients using Kaplan-Meier analysis. Results showed no significant difference in genotype distribution (Fig. 2) or IGF1R

Table V. Genotypic and allelic frequency of SNP rs2229765 in the *IGF1R* TK domain of NSCLC and control patients (age- and gender-matched).

Frequency	NSCLC patients (n=95), n (%)	Control patients (n=95), n (%)	P-value
Genotype			
AA	20 (21.0)	21 (22.1)	0.4351
GA	54 (56.8)	46 (48.4)	
GG	21 (22.1)	28 (29.4)	
Allele			
A	94 (49.4)	88 (46.3)	0.2636
G	96 (50.5)	102 (53.6)	

SNP, single-nucleotide polymorphisms; *IGF1R*, insulin-like growth factor 1 receptor; TK, tyrosine kinase; NSCLC, non-small cell lung cancer.

expression (Fig. 3A and B) in NSCLC patients. Analysis of genotype distribution in NSCLC patients showed that the P-value was 0.3564 while the P-value for trend was 0.1514 (Fig. 2). Analysis correlating IGF1R expression to genotype distribution in NSCLC patients showed that the P-value was 0.3005 while the P-value for trend was 0.7099 in patients with heterozygous genotype (Fig. 3A), and that the P-value was 0.1164 while the P-value for trend was 0.0277 for patients with homozygous dominant and homozygous recessive genotype (Fig. 3B).

Discussion

A number of previous studies have identified factors that may influence sensitivity to IGF1R inhibitors. Kim *et al* (35) evaluated the anti-proliferation effect of figitumumab in gastric and hepatocellular cancer cell lines and showed that the level of N-linked glycosylated IGF1R/IR heterodimeric receptor is highly associated with sensitivity to anti-IGF1R antibody in cancer cells. Another study demonstrated that IGF1R TK inhibitors (TKIs) exhibited significant antitumor activity in NSCLC cells with wild-type EGFR and KRAS compared to those with mutations in these genes (36).

Although SNPs have been reported in *IGF1R*, there is limited knowledge regarding the frequency of gene aberrations in the TK domain of this gene. Patients who harbour point mutations and deletions within exons 18-21 of the *EGFR* TK domain are known to have increased sensitivity to *EGFR* TKIs. Therefore, the mutation status of the *IGF1R* TK domain may also influence the binding of *IGF1R* TKIs to this receptor and therefore influence patient response to targeted therapy.

The present study investigated the frequency of gene aberrations in the *IGF1R* TK domain in NSCLC patients and whether such changes may influence IGF1R expression or survival rate. Initially, 100 NSCLC patients were screened for the presence of mutations and or deletions in exons 16-21 of the *IGF1R* TK domain. No non-synonymous SNPs or deletions were detected in any of the 100 patients screened. A synonymous SNP (rs2229765) was identified in the coding region of exon 16. In order to strengthen the power of the study, a further 98 NSCLC patients were screened for the presence of the SNP (rs2229765) and the frequency was compared to control disease-free individuals (n=866). No significance was found in the genotype (P=0.5487) or the allelic (P=0.9082) frequency (Table IV).

When patients were age- and gender-matched, no significance was identified in the genotype (P=0.4351) or the allelic (P=0.2636) frequency (Table V). The Kaplan-Meier survival analysis showed no significant survival advantage between the different genotypes in NSCLC patients (Fig. 2). There was also no significant difference when *IGF1R* expression was correlated to heterozygous genotype distribution in NSCLC patients. A significant difference for trend (P<0.05) was observed when IGF1R expression was correlated to combine the homozygous dominant and homozygous recessive genotype distribution in NSCLC patients (Fig. 3A and B).

The role of the SNP, rs2229765, has been examined in several diseases such as stroke breast cancer and type II diabetes. It is a synonymous mutation that encodes a change in the DNA sequence without altering the resultant protein sequence. These silent SNPs are presumed to be not significant but they may represent genetic markers for functional molecular alterations, as recent studies have revealed that through various mechanisms these synonymous SNPs may affect gene function and phenotype. Silent SNPs have been linked to >40 diseases that are a result of a genetic abnormality (37).

The possible role of this SNP has been investigated in numerous studies. According to FASTSNP, it is predicted that SNP rs2229765 may affect splicing regulation. It has been shown to affect the susceptibility to ischemic stroke in the Chinese population (38) and is associated with higher plasma concentrations of circulating IGF1R. In a study by Bonafè *et al* (39), polymorphic variants of the IGF1 response pathway genes, including IGF1R (G/A, codon 1013), phosphoinositol 3-kinase (T/C, 359 bp; A/G, 303 bp), insulin receptor substrate-1 (G/A, codon 972) and *FOXO1A* (T/C, 97,347 bp), were examined to observe whether they are involved in systemic IGF1 regulation and human longevity. It was found that subjects carrying at least an A allele at *IGF1R* have low free plasma IGF1 levels and live longer. A study performed in breast cancer identified that SNP rs2229765 had no association with breast cancer survival and that it appears to be a silent mutation (31). Therefore, thus far it has not been associated with any epidemiological traits. In another study, single SNP analysis revealed a significant association of SNP rs2229765 with percent and absolute mammographic density; increased numbers of the G allele increased the least squares means of mammographic density (40). The possible role of this polymorphism has also been examined in type II diabetes, which revealed no association with reduced birth weight, insulin sensitivity index or type II diabetes in a Danish population (41). These results also suggest that the rs2229765 polymorphism leads to a silent mutation.

A previous study investigated whether germline polymorphisms of the IGF1-pathway are associated with the response to cetuximab in wild-type KRAS drug-refractory metastatic colorectal cancer patients (mCRC) (28). Tissue samples from 130 drug-refractory mCRC patients enrolled in a phase II clinical trial of cetuximab monotherapy (IMC-0144) were used for the study. Analyses revealed that 5 IGF-pathway SNPs were

significantly associated with progression-free survival and/or OS. Patients harbouring the *IGF1* rs2946834 A/A genotype had a 50% overall response rate, while patients with the A/G genotype had 0%. This indicates that IGF1-pathway polymorphisms may predict cetuximab efficacy in wild-type KRAS mCRC patients.

Identifying functional polymorphisms in *IGF1R* and its pathway could be used to select patients that would benefit from IGF1R-targeted therapy resulting in more accurate treatment for individuals with improved effectiveness and reduced toxicities. In the present cohort of 100 NSCLC patients, no non-synonymous SNPs were detected in the IGF1R TK domain. There was no significant association between SNP rs2229765 and *IGF1R* expression or patient survival. This data indicates that the *IGF1R* TK domain does not appear to be as susceptible to mutations as *EGFR*. Therefore, as opposed to *EGFR*, it will not be necessary to screen for mutations in *IGF1R* to predict response to targeted therapy.

Acknowledgements

Genetic data on the control samples were generated by the International Schizophrenia Consortium.

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