Pharmacogenetics of EGFR and VEGF inhibition

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Even though treatment of several types of solid tumours has improved in the past few years with the introduction of the monoclonal antibodies against epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF), response rates to these targeted therapies are modest. Pharmacogenetic factors have the potential to select patients with higher chance of response to agents that target these pathways. This review provides an overview over germ-line variations in genes that are potentially involved in the pharmacodynamics of the monoclonal antibodies cetuximab, panitumumab and bevacizumab, and which may underlie variable anti-tumour response.

The treatment of solid tumours has changed in the past five years with the introduction of monoclonal antibody (MAb) drugs targeting growth factor pathways that are crucial for tumour growth and invasiveness. The epidermal growth factor receptor (EGFR) targeting MAbs cetuximab and panitumumab and the vascular endothelial growth factor (VEGF) targeting MAb bevacizumab are approved for the treatment of metastasized colorectal cancer (mCRC). Cetuximab and bevacizumab are also approved for the treatment of advanced squamous cell carcinoma of the head and neck (SCCHN) and advanced non-squamous, non-small cell lung cancer (NSCLC), respectively. These MAbs are commonly administered in combination with first-line chemotherapy, whereas monotherapy is also applied in subsequent lines of therapy.

Despite overall improving cancer treatment, the addition of these MAbs to chemotherapy increases response rates by only 10–20% [1–3] and adverse events such as moderate to severe rash for the EGFR inhibitors and gastro-intestinal perforations and hypertension for bevacizumab are relatively common [1–3]. Moreover, the introduction of these MAbs has almost doubled the cost of treatment [4].

Therefore, selection of patients for treatment based on predictive factors for response, survival and/or toxicity could improve treatment success as well as cost-effectiveness.

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Pharmacogenetics is aimed at understanding and predicting an individual's drug response based upon genetic variation. Whereas somatic mutations occur only in the affected organ or disease locus (tumour) and result in a different genetic composition of a tumour compared with other tissues in the body, germ-line polymorphisms have an ancestral origin and are heritable. In this review, we give an overview of heritable genetic factors that might predict drug-induced anti-tumour response and toxicity of EGFR and VEGF targeting MAbs, based upon candidate genes for these pathways. Also, we give an overview of pharmacogenetic studies with drugs that target these pathways.

Epidermal growth factor (EGF) pathway

Cetuximab is a chimeric mouse/human IgG_1-type MAb, whereas panitumumab is a fully human IgG_2-type MAb. Both MAbs bind specifically to the extracellular domain of the EGFR and are competitive inhibitors of the natural ligands EGF and transforming growth factor-α (TGFα).

The small G protein k-ras, the protein kinase b-raf and phosphoinositide 3-kinase (encoded by *KRAS*, *BRAF* and *PIK3CA*, respectively) play a central role as intracellular mediators of EGFR signalling [5], ultimately leading to induced transcription of several factors including interleukin-8 (IL8), VEGF and cyclin D1 (CD1, coded by *CCND1*). Cyclooxygenase-2 (COX2, encoded by *PTGS2*) is an upstream mediator of EGFR activity, presumably through the effect of prostaglandin E_2 (Fig. 1).

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GLOSSARY

SNP (single nucleotide polymorphism): a change of a single base of germ-line DNA, as compared with wild-type, which occurs in ≥1% of the population. Because any individual carries two alleles, the SNP can be present in both alleles (homozygote), on only one allele (heterozygote) or not at all (wild-type).
CNP (copy number polymorphism): in contrast to a SNP, a CNP encompasses ≥1000 base pairs or more. Regarding heritability and population frequency, the definitions are the same.
Mutation: a change in DNA that occurs either very infrequently (≤1% in the population), or only in an affected organ. In the latter case, the mutation is not inherited.
Haplotype block: SNPs are naturally inherited in neighbouring clusters, which are called haplotype blocks.
ADCC (antibody-dependent cell-mediated cytotoxicity): the recognition by natural killer cells of the Fc region of an antibody after binding to the antigen, followed by killing of the antigen-presenting cell.
Prognostic factor: a marker for prognosis of a disease, not related to treatment.
Predictive factor: a marker for response to a certain treatment.

Heritable genetic variants in genes in the EGF pathway will be discussed below (Table 1).

Epidermal growth factor receptor (EGFR)

The first intron of the *EGFR* gene has an important regulatory function and contains a heritable polymorphic microsatellite sequence of 9–23 CA repeats. Most common alleles are the 16-repeat allele in Caucasians and Afro-Americans, and the 20-repeat in Asians [6,7]. There is good to complete (93–100%) similarity of this polymorphism between normal and tumour tissue [8–10], which is reassuring because the *EGFR* gene is highly sensitive to somatic alteration through loss of heterozygosity, mutations or copy number alterations. A higher number of CA repeats is associated with decreased expression of EGFR on both mRNA and protein level *in vitro* [10,11], but this association was not consistently found *in vivo* [9,12].

Two single nucleotide polymorphisms (SNPs; see glossary box) in the promoter (−216G>T and −191C>A) are both associated with increased expression of EGFR [7,13]. A nonsynonymous SNP (1808G>A) in the extracellular domain of EGFR results in lower binding-affinity of EGF and TGFα and attenuated growth response to these growth factors *in vitro* [14].

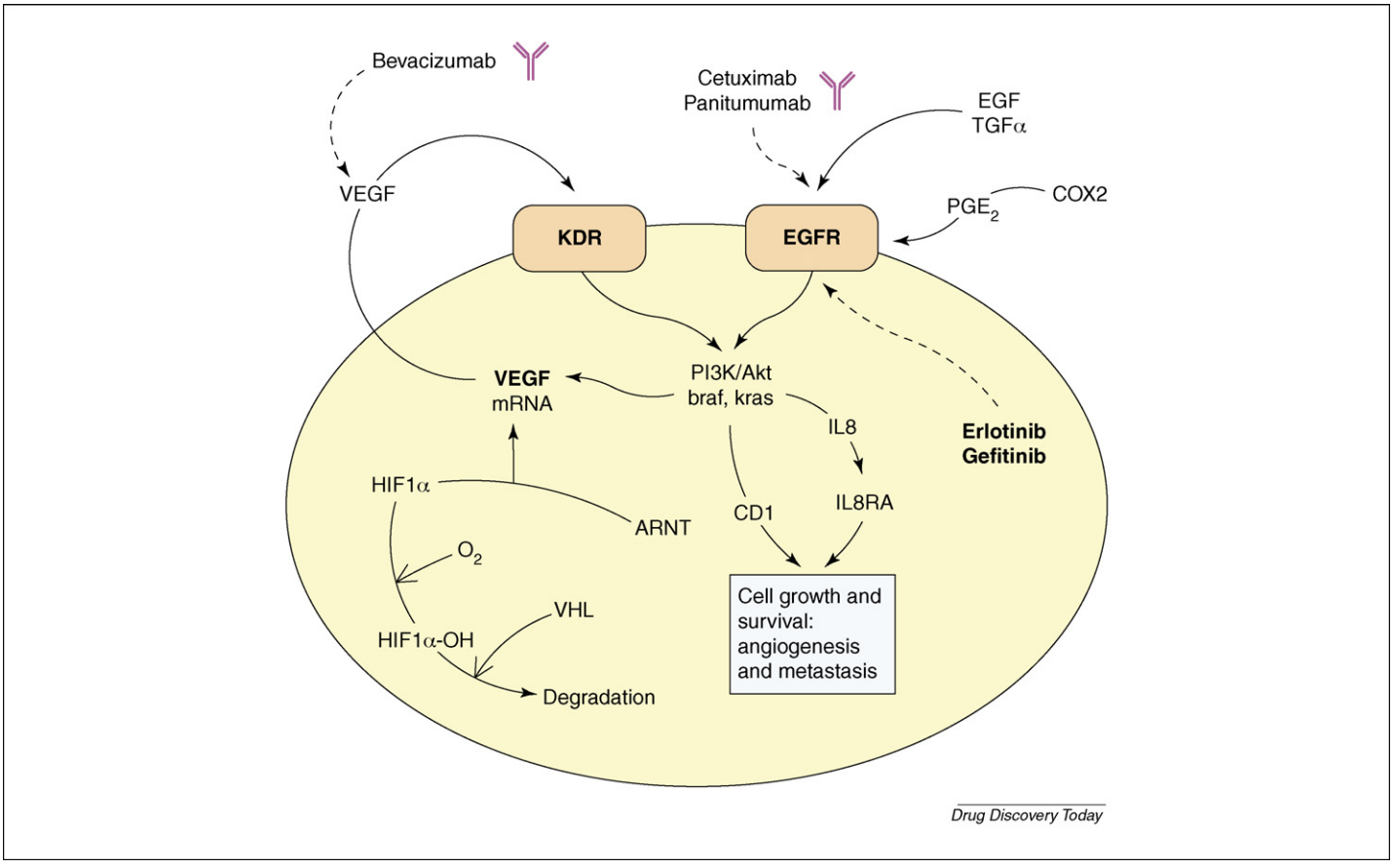

FIGURE 1

Simplified overview of the EGF and VEGF pathways. *Abbreviations* – ARNT: aryl hydrocarbon receptor nuclear translocator; braf: protein kinase b-raf; CD1: cyclin D1; COX2: cyclooxygenase 2; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; HIF1α: hypoxia inducible factor 1α; HIF1α-OH: hydroxylated hypoxia inducible factor 1α; IL8: interleukin 8; IL8RA: interleukin 8 receptor A; KDR: kinase domain receptor; kras: small G protein k-ras; PGE_2: prostaglandin E_2; PI3K/Akt: phosphoinositide 3-kinase/akt protein kinase; TGFα: transforming growth factor α; VEGF: vascular endothelial growth factor; VHL: von Hippel-Landau tumour suppressor.

TABLE 1

Overview of polymorphisms in genes that code for enzymes involved in the EGFR pathway

Enzyme (gene)	Polymorphism	Phenotype	Function	Refs	Pharmacogenetic association	Refs
EGFR (*EGFR*)	−191C>A		Higher EGFR	[7,13]		
	−216G>T		Higher EGFR	[7,13]	Gefitinib (NSCLC): carriage of −216T allele → longer PFS	[15]
	$(CA)_{9-23}$		Higher EGFR for lower amount of CA repeats	[10,11]	Gefitinib (NSCLC): low amount of CA repeats → higher response and TTP and PFS	[9,15]
	1808G>A	Arg497Lys	Unknown		Monotherapy cetuximab (mCRC): heterozygotes → longer PFS	[18]
EGF (*EGF*)	61A>G		Higher EGF	[16,17]	• Monotherapy cetuximab (mCRC): simultaneous carriage of 61A and *CCND1* 870G allele → longer OS • Monotherapy cetuximab (mCRC): 61GG homozygotes → longer PFS	[18,28]
COX2 (*PTGS2*)	−765G>C		Lower COX2	[19,20]	Monotherapy cetuximab (mCRC): −765CC homozygotes → longer PFS	[18]
IL8 (*IL8*)	−251T>A		Higher IL8	[25]		
IL8RA (*CXCR1*)	2607G>C	Ser276Thr	Unknown			
CD1 (*CCND1*)	870G>A		Higher CD1	[27]	Monotherapy cetuximab (mCRC): carriage of 870G allele → longer OS	[28]

Recently, two pharmacogenetic studies were published on the sensitivity of NSCLC patients to the EGFR tyrosine kinase inhibitor (TKI) gefitinib. These studies are important examples of the utility of pharmacogenetics for EGFR inhibitors.

Han *et al*. reported an increased response and time to progression (TTP) for patients with ≤37 CA repeats in Korean NSCLC patients, regardless of the presence of somatic mutations [9].

Similarly, NSCLC patients of predominantly Caucasian origin, who were homozygous for two short CA-repeat alleles (defined as ≤16 CA repeats per allele) had better progression-free survival (PFS) compared with carriers of at least one allele with >17 CA repeats. Also, patients who carried the −216T allele had longer PFS. Patients who were homozygous for the short CA-repeat allele and simultaneously carrier of the −216T allele, had improved PFS and overall survival (OS) [15].

In line with these findings, Amador *et al*. reported higher sensitivity to another EGFR inhibiting TKI, erlotinib, in cell lines with ≤35 CA repeats compared with cell lines with >35 repeats. Also, the authors found increased incidence of skin toxicity in gefitinib treated CRC patients with ≤35 CA repeats [10].

Upstream regulators of EGFR

A SNP in the 5′-UTR of the *EGF* gene (61A>G) has been associated with higher EGF protein expression *in vitro* [16] and *in vivo* [17]. The 61GG genotype was associated with increased PFS in mCRC patients who were treated with cetuximab monotherapy [18].

A functional SNP in the promoter region of the *PTGS2* gene (−765G>C) has been associated with lower promoter activity *in vitro* [19] and with lower expression of the *PTGS2* gene product, COX2 *in vivo* [20]. Illustrative of its function is the strong association with decreased risk of myocardial infarction and stroke for the −765C allele [21]. Recently, an association for increased PFS for the −765CC genotype was reported in mCRC patients treated with single agent cetuximab [18].

Downstream signalling

The presence of somatic mutations in *KRAS*, but not in *BRAF* and *PIK3CA*, has been associated with decreased effect of cetuximab in CRC patients [22,23], though not unequivocally [24]. However, no reports are available on heritable polymorphisms in these genes.

A SNP in the 5′-UTR of the *IL8* gene (−251T>A) has been associated with increased IL8 production [25]. The IL8 receptor alpha, IL8RA (encoded by the gene *CXCR1*) contains a nonsynonymous SNP in exon 2 (2607G>C) [26], whose function remains unclear. A SNP in the *CCND1* gene (870G>A) has been associated with higher expression of CD1 [27].

Zhang *et al*. investigated whether there was an association for the polymorphisms in the *CCND1* (870A>G), *PTGS2* (−765G>C), *EGF* (61A>G), *EGFR* (1808G>A and CA repeats), *IL8* (−251T>A) and *VEGF* (+936C>T) genes with the effect of cetuximab given as a single agent in advanced CRC patients [28]. Homozygotes for the *CCND1* 870A allele had a shorter OS compared with carriers of the 870G allele [28]. In combined analysis, patients who carried both a *CCND1* 870G allele and an *EGF* 61A allele had longer OS, whereas the other polymorphisms were not associated with survival [28]. These results, though valuable, need to be interpreted with care, as this was an exploratory study. The fact that seven different polymorphisms were analyzed in a small population raises the probability of false positive associations. However, together with the other association studies of the EGF pathway, these findings provide an important starting point for adequately powered confirmation studies.

Vascular endothelial growth factor (VEGF) pathway

Bevacizumab is a humanized IgG1-type MAb directed against soluble VEGF, one of the key moderators in angiogenesis, which is thought to be important for tumour growth and invasiveness [29]. VEGF exerts its pro-angiogenic effect via VEGF receptor-2, a tyrosine kinase receptor that is also referred to as kinase insert

TABLE 2

Overview of polymorphisms in genes that code for enzymes involved in the VEGF pathway

Enzyme (gene)	Polymorphism	Phenotype	Function	Refs
VEGF (*VEGF*)	−2578C>A		Lower or higher VEGF	[34,35]
	−1154G>A		Lower VEGF	[34,35]
	−460C>T		Increased promoter activity	[36]
	+405G>C		Lower or higher VEGF	[31,34,37]
	+936C>T		Lower VEGF	[32,33]
KDR (*KDR*)	$+4422(AC)_{11-14}$		11 CA repeats higher promoter activity than 12 CA repeats	[42]
HIF1α (*HIF1A*)	1772C>T	Pro582Ser	Higher HIF1α	[43–46]
	1790G>A	Ala588Thr	Higher HIF1α	[43]

domain receptor (KDR). Transcription of VEGF is regulated by hypoxia inducible factor-1α (HIF1α) (Fig. 1).

Till date, five functional SNPs in the 5′ and 3′ regions of the *VEGF* gene have been described (Table 2) [30–32].

The variant alleles of the −1154G>A and +936C>T SNPs are associated with lower VEGF production [32–35], whereas the variant allele of the −460C>T SNP results in increased promoter activity [36]. There is less agreement on the functionality of the −2578C>A and +405G>C SNPs, as both increased and decreased VEGF production have been reported [31,34,35,37].

It must be noted though that the above-mentioned SNPs are inherited in clusters in so-called haplotype blocks (see glossary box) [31,35,36,38–41]. It is likely that only one SNP is truly functional with regard to VEGF expression, whereas the others are merely proxies for this one. This truly causal SNP, however, has so far not been identified.

There are several nonsynonymous SNPs in the coding region of the *KDR* gene (see http://www.ncbi.nlm.nih.gov/projects/SNP). Nonetheless, only functionality of a CA-repeat polymorphism in intron 2 of the *KDR* gene ($+4422(AC)_{11-14}$) has been determined. The 11-repeat polymorphism results in higher promoter activity *in vitro* [42]. Even though the 11- and 12-repeat alleles were most common in the Japanese population, the allele frequencies in other populations are unknown.

The nonsynonymous SNP 1772C>T in the gene encoding HIF1α (*HIF1A*) has been associated with increased expression of HIF1α [43–45]. The enzyme coded by the variant allele is also less sensitive to hydroxylation-dependent degradation [46], which results in further increased protein levels. Another nonsynonymous SNP (1790G>A) in the *HIF1A* gene has also been associated with increased HIF1α expression [43].

As these SNPs result in increased abundance of the HIF1α protein, it is expected that the SNPs ultimately result in increased VEGF expression (Fig. 1). The relationship between the *HIF1A* SNPs and VEGF mRNA levels has been demonstrated, whereas no relationship with VEGF protein expression was found [45,47].

ARNT is most commonly described as a subunit of aryl hydrocarbon receptor (AHR), which induces transcription of the cytochrome P450 isozyme CYP1A1 in response to exogenous stimuli such as cyclic aromatic hydrocarbons from cigarette smoke. As part of a dimer with HIF1α, ARNT induces VEGF transcription. Genetic variation of the ARNT gene may therefore be of importance for VEGF production. However, till date no functional polymorphisms in the *ARNT* gene have been described.

Up to now, no pharmacogenetic studies have been published for agents that target the VEGF pathway. The publication of these studies is eagerly awaited, as they will provide a foundation for further, hypothesis testing research for this pathway.

Polymorphisms for MAbs in general

The plasma half-life of MAbs is generally relatively long: the half-life of bevacizumab (20 days) is similar to that of endogenous IgG_1, whereas the half-life of cetuximab and panitumumab is 70–100 hours and 7.5 days, respectively. The shorter half-life of the latter MAbs can in part be explained by internalization and degradation of the receptor–MAb complex after binding. It is postulated

TABLE 3

Overview of polymorphisms in genes that code for enzymes involved in the mechanism of action of IgG type monoclonal antibodies

Enzyme (gene)	Polymorphism	Phenotype	Function	Refs	Pharmacogenetic association	Refs
FcRn (*FCGRT*)	VNTR1-5		VNTR3 higher FcRn than VNTR2	[49]		
FcγR2A (*FCGR2A*)	559T>G	His131Arg	Unknown		• Rituximab (lymphoma): carriage of 131Arg allele → lower response and PFS • Cetuximab (mCRC): 131Arg homozygote → lower response and PFS	[54,57]
FcγR3A (*FCGR3A*)	535A>G	Phe158Val	Higher affinity for IgG_1	[50,51]	• Rituximab (lymphoma): carriage of 158Phe allele → lower response and PFS • Infliximab (mCrohn): 158Val allele → increased biological response • Cetuximab (mCRC): 158Val homozygote → lower response and PFS	[53–57]

that antibodies of the IgG type, such as bevacizumab, are protected from degradation by the neonatal Fc receptor (FcRn, coded by *FCGRT*) [48].

Sachs *et al*. recently described a variable number of tandem repeats (VNTR) within the promoter of the *FCGRT* gene consisting of five different alleles with one to five repeats (VNTR 1–5) [49]. The allele frequencies of VNTR2 and VNTR3 were 0.075 and 0.92, respectively in Caucasians. The VNTR3 allele was associated with higher FcRn expression both *in vitro* and *in vivo*. Also, binding of IgG was higher among VNTR3 homozygotes compared with VNTR2/VNTR3 heterozygotes [49]. Possibly, individuals carrying the VNTR3 allele have prolonged plasma half-life of bevacizumab, and even increased response.

Cetuximab is a competitive inhibitor of EGFR, which results in decreased utilization of this pathway. As cetuximab is of the IgG_1 type, it is likely that antibody-dependent cell-mediated cytotoxicity (ADCC) also plays a role in its mechanism of action. The Fc region of the antibody can be recognized by Fcγ-receptors on cytotoxic immune effector cells such as natural killer cells and macrophages. Two activating Fcγ-receptors are CD16A and CD32A (encoded by respectively *FCGR3A* and *FCGR2A*) are polymorphic (Table 3).

A SNP in the *FCGR3A* gene (559T>G; Phe158Val) has been studied since the early 1990s. IgG_1 binding is higher for the 158Val allele, which results in increased activation of ADCC but not in altered expression [50,51]. Also, the affinity of the IgG_1-type MAb against CD20 expressing B cells, rituximab, was highest for the 158Val allele [52]. It is therefore not surprising that response and PFS to rituximab in follicular lymphoma was higher for homozygotes of the 158Val allele, compared with carriers of the 158Phe allele [53,54]. This SNP was not associated with clinical response to another IgG_1 MAb against TNFα, infliximab, in Crohn's disease, but increased biological response (decrease in C-reactive protein) was associated with the 158Val allele [55,56].

Even though the function of a SNP in the *FCGR2A* gene (535A>G; His131Arg) with regard to IgG_1 has not been established, an association with worse response and PFS to rituximab in follicular lymphoma was found [54]. However, this association was not confirmed by another study [53].

Very recently, Zhang *et al*. showed that mCRC patients treated with cetuximab monotherapy, who were homozygous for either the *FCGR2A* 131Arg or *FCGR3A* 158Val allele had shorter PFS and decreased response [57]. The reason for this result, which for *FCGR3A* is opposite to what would be expected, is not known. A possible explanation is that copy number polymorphism (CNP) (see next paragraph) at the locus of *FCGR3A* plays a role. This, however, has not been investigated. It is also probable that this finding is a false positive discovery, because not only these two genotypes have been investigated but also seven others in a previous analysis of the data [28].

Even though ADCC does not play a role for bevacizumab, it is likely that its effect is modified by similar mechanisms.

Copy number polymorphisms

An interesting novel field of pharmacogenetic research includes heritable variation of copy number of DNA segments of 1 kb or larger of the genome [58]. Analogous to the definition of a SNP, a CNP is a structural variant that occurs at a frequency of >1% in the population. Since the first whole genome array studies of this phenomenon were published in 2004 [59,60], an open-access online database has been developed in which structural variations of the human genome are assembled [60,61] (see http://projects.tcag.ca/variation).

Several studies have demonstrated that increased intratumoural EGFR copy number in advanced CRC patients is associated with effectiveness of cetuximab [22,24,62]. However, is must be noted that this is a somatic phenomenon, which is probably involved in the aetiology of the tumour. In the Database of Genomic Variants, there are no CNPs on the EGFR locus. Also, no CNPs are reported at the loci that cover the *TGFA*, *IL8*, *CXCR1*, *BRAF*, *KRAS*, *PIK3CA*, *PTGS2*, *VEGF*, *KDR*, *HIF1A* or *ARNT* genes. There is an infrequent CNP in the *EGF* gene (1 reference to loss of the locus in 36 subjects), but CNPs on the locus that covers the *CCND1* gene occurs in 6 of 95 subjects. Also, the locus that contains the *FCGRT* gene shows heritable loss at a frequency of approximately 0.26. There is also considerable CNP covering the *FCGR2A* and *FCGR3A* genes, with equal amount of gain and loss of this locus. Illustrative of the influence of copy number variation was recently published, showing that copy number variation of the *FCGR2A* and *FCGR3A* containing region (that also contains the *FCGR3B* gene encoding CD16B) was associated with susceptibility to systemic autoimmune diseases [63].

Discussion

It is accepted that germ-line polymorphisms (both SNPs and CNPs) have the potential to predict outcome of therapy. Predicting outcome of therapy with cetuximab, panitumumab and bevacizumab is especially warranted, as response rates are moderate, with possible serious adverse events, at high financial cost. In this review, we give an overview of studies on polymorphisms in candidate genes in the EGF and VEGF pathways.

Till date, only few small studies have shown an association of genetic polymorphisms in genes of the EGF pathway with response to EGFR targeting therapies (Table 1), whereas studies for the VEGF pathway are thus far lacking.

However, these studies do not provide sufficient evidence to routinely genotype patients before applying these therapies. The associations need to be confirmed in one or more sufficiently powered prospective studies first. A requirement for these studies is the presence of a control group without the treatment of interest. Only then a distinction between predictive and prognostic factors (see glossary box) can be established. This latter point is of great importance, because numerous studies have shown an association of polymorphisms within genes in the EGF and VEGF pathways with the risk and progression of several types of cancer [64].

Based upon these considerations and available studies, the predictive value of *FCGR2A*, *FCGR3A*, *EGF* and *CCND1* genotyping should be investigated prospectively for cetuximab in cases and controls. For bevacizumab and panitumumab, hypothesis generating association studies, based upon the candidate genes in this review, are required for further research.

In any pharmacogenetic association study, confounders must be carefully corrected for, in order to find independent predictive factors. Factors that need to be taken into account are gender and race, as these can impact on the response to therapy. Moreover,

allele frequencies are usually different among populations. Also, care should be taken to reduce the chance of false positive associations when testing multiple genotypes. This can be accomplished by adjusting the level of significance based upon the number of genotypes tested, for example with the Bonferroni correction.

Interestingly, there appears to be a major interplay between these two pathways. For example, higher intratumoural VEGF levels were associated with resistance to single agent cetuximab in mCRC patients [65]. Moreover, the combination of cetuximab and irinotecan in mCRC patients reduced circulating VEGF levels, and of these patients with the most prominent decrease of VEGF responded better as indicated by TTP and OS compared with patients who showed only a modest reduction in VEGF levels [66]. Therefore, it makes sense to look at multiple SNPs and CNPs within both pathways simultaneously.

Finally, true usefulness of a predictive marker can only be assessed with the application of a validated predictive test in a prospective setting. The test should allocate different treatment options for patients with the genotype of interest and solid endpoints should be investigated.

In conclusion, pharmacogenetics (including germ-line SNPs and CNPs) of EGFR and VEGF inhibitors will most probably find its way to daily clinical practice, provided that the above suggestions for future research have been met.

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