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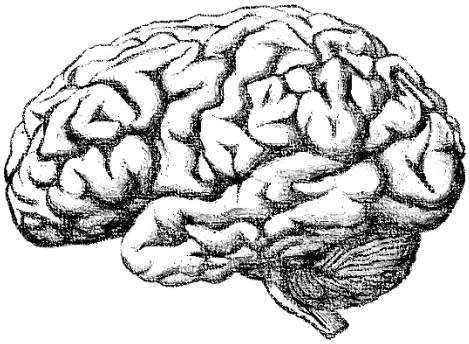
Prediction of bevacizumab response and resistance in glioblastoma patients



PhD thesis

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Department of Radiation Biology

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Thesis title

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PREFACE

The present Ph.D. thesis “Prediction of bevacizumab response and resistance in glioblastoma patients” is submitted in order to achieve the Ph.D. degree at the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, April 1st, 2016.
The work presented in the thesis was carried out at the Department of Radiation Biology, Department of Oncology, Rigshospitalet, Copenhagen, Denmark.

The result section of this thesis comprises one paper published in *Acta Oncologica* (Study I) and two manuscripts submitted for publication in *Molecular Oncology* (Study II) and *International Journal of Cancer* (Study III).

Thomas Urup
Copenhagen, April 2016

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The work presented in this thesis was performed at the Department of Radiation Biology. Here, I have been dependent on all colleagues. Through strong friendships we have established an open-minded scientific atmosphere, perfect for new discoveries which will improve the treatment of glioblastoma patients. I thank Signe Regner Michaelsen for your great scientific support in all my projects. I want to thank Kirsten Grunnet for introducing me to our clinical databases and for keeping them updated. Mette Villingshøj for running the laboratory and for your unyielding positive attitude. Ole Didrik Lærum, I want to thank you for your book “Den forunderlige vitenskapen”, which I have read with great joy. I also want to thank all the medical students who have performed impressive scientific work. Specifically, I want to thank Anders Toft who has been an indispensable help for me and I truly hope we will continue our collaboration for many years to come.

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CONTENTS

i. LIST OF ABBREVIATIONS

BEV	bevacizumab
CCNU	lomustine
EGFR	epidermal growth factor receptor
FFPE	formalin-fixed, paraffin-embedded
FLAIR	fluid-attenuated inversion recovery
HLA	human leucocyte antigen
IDH1	isocitrate dehydrogenase 1
IPA	Ingenuity pathway analysis software
IRI	irinotecan
MGMT	O^6-methylguanine-DNA methyltransferase
MMP2	metalloproteinase 2
MRI	magnetic resonance imaging
NA	not available
NF1	neurofibromin 1
ORR	overall response rate
OS	overall survival
PDGFRA	platelet-derived growth factor alpha gene
PET	positron emission tomography
PFS	progression-free survival
PFS6	progression-free survival rate at 6 months
PS	performance status
PTEN	phosphatase and tensin homolog
RANO	revised assessment in neuro-oncology
RNA	ribonucleic acid
RT	radiotherapy
TGF-β	transforming growth factor beta
TMZ	temozolomide
VEGF	vascular endothelial growth factor A
VEGFR	vascular endothelial growth factor receptor
WHO	world health organization

ii. SUMMARY

Glioblastoma is the most common and malignant primary brain tumor in adults. Despite aggressive treatment, including surgery and radiotherapy in combination with chemotherapy, newly diagnosed patients have a median survival of less than 15 months. At tumor recurrence most known treatment modalities have limited clinical effect.

Glioblastoma is characterized by increased development of abnormal blood vessels, which is associated with aggressive tumor growth. This emphasizes the potential value of targeting the vasculature. Clinical studies have shown that bevacizumab, an anti-angiogenic agent, in combination with chemotherapy produces clinical response in approximately 25-30% of recurrent glioblastoma patients. This group of patients has demonstrated improved survival as well as quality of life, highlighting the importance of identifying patients who will benefit from bevacizumab combination therapy. Currently, no validated predictive biomarkers for bevacizumab response have been identified.

The overall objective of this thesis was to identify predictive biomarkers for bevacizumab response in recurrent glioblastoma patients. The thesis is based on the following three studies:

Study I

The aim of this study was to identify predictive and prognostic clinical factors. A total of 219 recurrent glioblastoma patients treated with bevacizumab and irinotecan therapy were included in the study. Multiple factors were screened for association with response, progression-free survival (PFS) and overall survival (OS). Three independent prognostic factors were associated with reduced PFS and OS: Corticosteroid use, multifocal disease and neurocognitive deficit. Based on these factors a prognostic model for OS was established. The model was validated in an independent cohort of 85 patients and can therefore be used in clinical practice for treatment planning.

Study II

This study aimed to identify predictive biomarkers for bevacizumab response. A total of 82 recurrent glioblastoma patients treated with bevacizumab combination therapy were included. Archived tumor tissue samples from the time of glioblastoma diagnosis were gene expression profiled by a method covering 800 genes (NanoString). By comparing gene profiles with response data, we identified two predictive genes for bevacizumab response: Low gene expression of

angiotensinogen and high expression of a human leucocyte antigen class II gene (HLA-class II gene, *HLA-DQA1*). Based on these two genes we established a predictive model for bevacizumab response.

Study III

The aim of this study was to investigate the response and resistance mechanisms related to bevacizumab therapy. The study included 21 recurrent glioblastoma patients who had accessible archived tumor tissue from both before and after bevacizumab treatment. Gene expression profiles of paired tumor tissue samples were generated by means of the RNA-sequencing method. It was found that bevacizumab combination therapy produces a significant change in the gene profile of responders, but almost no change in non-responders. This suggests that responding tumors adaptively respond or progress while non-responding glioblastomas progress unaffected by the treatment. To uncover potential response and resistance mechanisms the gene changes were analyzed by functional data mining based on published data.

Conclusion

Our findings have generated new knowledge concerning which recurrent glioblastoma patients will benefit from bevacizumab treatment. We have found that some patients are unlikely to profit from treatment due to a poor prognosis. In addition, we have established a predictive model for bevacizumab response. If the model can be validated it can be used to optimize and individualize the treatment of recurrent glioblastoma patients. We have expanded upon the existing knowledge regarding bevacizumab response and resistance mechanisms. This can be used in order to establish new and more effective bevacizumab combination treatments for glioblastoma patients.

iii. RESUMÉ

Glioblastom er den hyppigste og mest aggressive form for hjernekræft hos voksne. På trods af intensiv behandling bestående af kirurgi og strålebehandling, kombineret med kemoterapi, har glioblastompatienter en gennemsnitlig overlevelse på mindre end 15 måneder. Når sygdommen recidiverer, har de fleste behandlingsformer vist begrænset effekt.

Glioblastom er karakteriseret ved udtalt nydannelse af abnorme blodkar, hvilket giver kræftcellerne vækstfordele. Derfor er bevacizumabbehandling, der målrettet hæmmer nydannelsen af blodkar, blevet fremsat som en effektiv behandlingsstrategi. Kliniske studier har vist, at ca. 25-30% af glioblastompatienter med tilbagefald responderer på behandling med bevacizumab i kombination med kemoterapi. Patienter, som opnår dette behandlingsrespons, lever længere og har bedre livskvalitet. Dette understreger vigtigheden af at identificere de patienter, som vil få gavn af bevacizumab-kombinationsbehandling. I dag findes der ingen prædiktive biomarkører, der kan forudsige, om en patient vil få effekt af bevacizumab-behandling.

Det overordnede formål med denne Ph.D. afhandling var derfor at identificere biomarkører, som kan forudsige bevacizumab-behandlingsrespons hos patienter med tilbagefald af glioblastom. Afhandlingen er baseret på følgende tre studier:

Studie I

Formålet med dette studie var at identificere prædiktive og prognostiske kliniske faktorer. I alt blev der inkluderet 219 patienter med glioblastom, som ved tilbagefald blev behandlet med bevacizumab og kemoterapi. Flere forskellige relevante faktorer blev screenet for association med respons og overlevelse. Tre prognostiske faktorer associeret med en ringe overlevelse blev identificeret: Brug af binyrebarkhormon, multifokal sygdom og neurokognitive symptomer. De tre faktorer blev brugt til at etablere en prognostisk model for overlevelse. Modellen blev valideret i en uafhængig patient kohorte og kan hermed bruges i klinikken til behandlingsplanlægning.

Studie II

I dette studie var formålet at identificere prædiktive biomarkører for respons på bevacizumab-kombinationsbehandling blandt 82 patienter med recidiverende glioblastom. Genprofiler på arkiveret tumorvæv blev genereret med en metode, der analyserede 800 udvalgte gener (NanoString). Ved at sammenholde genprofilerne med responsdata fandt vi to prædiktive gener for bevacizumab-respons: Lavt genudtryk af angiotensinogen og højt udtryk af et humant leukocyt

antigen klasse II gen. Baseret på de to gener etablerede vi en klinisk anvendelig, prædiktiv model for bevacizumab respons.

Studie III

Studiets formål var at undersøge respons- og resistens-mekanismer associeret med bevacizumab-behandling. Studiet inkluderede 21 glioblastompatienter, som havde tilgængeligt, arkiveret tumorvæv fra før og efter bevacizumabbehandling. Genprofilering af tumorvæv blev genereret med metoden RNA-sequencing. Vi fandt markante genudtryksændringer i bevacizumab-responderende glioblastomer og næsten ingen ændringer i ikke-responderende glioblastomer. Dette resultat indikerer, at responderende glioblastomer adapterer til behandlingen via respons eller progression, mens ikke-responderende glioblastomer vokser upåvirket videre under behandlingen. Ved at sammenholde genændringerne med publiceret litteratur, forsøgte vi at afdække potentielle respons- og resistensmekanismer.

Konklusion

Vores resultater har genereret ny viden, om hvilke glioblastompatienter, som har effekt af bevacizumabbehandling ved sygdomstilbagefald. Vi har fundet, at en gruppe af patienterne med stor sandsynlighed ikke vil have gavn af behandlingen grundet en ringe prognose. Derudover har vi etableret en prædiktiv model for bevacizumabrespons. Hvis modellen kan valideres, kan den bruges til at optimere og individualisere behandlingen af glioblastompatienter. Vi har ydermere dannet ny viden om respons- og resistens-mekanismer ved bevacizumabbehandling. Dette kan anvendes med henblik på at etablere nye og mere effektive bevacizumab-kombinationsbehandlinger til glioblastompatienter.

1. INTRODUCTION

1.1 Glioblastoma

Gliomas are of neuroepithelial origin and according to the WHO classification three main histological types have been defined: Oligodendroglioma, mixed oligoastrocytoma and astrocytoma. These are histologically graded by considering four key features: nuclear atypia, mitosis, microvascular proliferation and necrosis. The most malignant is grade IV glioma, termed glioblastoma, which separates from the lower grade gliomas by expressing necrosis and/or microvascular proliferation.[1]

Glioblastoma represents approximately 70% of gliomas and is the most common primary malignant brain tumor, affecting more than 3/100,000 individuals per year in western countries.[2;3] They arise either *de novo* as primary glioblastoma (95%) or develop from pre-existing lower grade gliomas. Most primary glioblastomas manifest in elderly patients, while secondary glioblastomas commonly affect younger patients prior to the age of 45 years.[3] The etiology of gliomas is largely unknown.[4]

The current standard treatment of newly diagnosed glioblastoma patients includes maximal safe resection, radiotherapy plus concomitant and adjuvant alkylating chemotherapy (temozolomide). This treatment was established in 2005 based on a phase-III trial demonstrating improved median overall survival (OS) (14.6 vs 12.1 months) and improved two-years survival rate (27% vs. 10%) by addition of temozolomide during and after radiotherapy compared to radiotherapy alone.[5] In clinical practice prognostic models have been used for selection of patients who will most likely benefit from standard thereapy.[6] However, with the recent implementation of the O^6-methylguanine-DNA methyltransferase (*MGMT*) gene promoter methylation status, a predictive biomarker for temozolomide benefit,[7;8] glioblastoma treatment has become more individualized.

1.2 Recurrent glioblastoma

Most cases of glioblastoma recur after standard therapy and no standard treatment in the recurrent setting has been established. Despite numerous agents having been tested, most have failed in early clinical trials.[9;10] This may be explained by the inherent poor prognosis of these

patients, in addition to patient and tumor heterogeneity. These factors complicate the design of clinical trials and the identification of effective treatments.

1.3 Patient heterogeneity

Glioblastoma patients vary in age, general well-being (performance status) and other classical oncological prognostic factors. Furthermore, different anatomical tumor locations produce a wide spectrum of more or less severe neurological impairments. Glioblastoma is a systemic brain disease, and non-measurable invasive tumor cells may also contribute to the clinical variation between patients. Additionally, tumors can be located in or near vital brain areas, complicating surgical resection and radiotherapy planning. Accordingly, glioblastoma patients differ profoundly in their clinical presentation before and during treatment. This complicates the interpretation of clinical outcome, especially in terms of prognosis and quality of life.

1.4 Tumor heterogeneity

1.4.1 Morphology

Histologically the tumors consist of heterogeneous cell populations and extracellular matrix components. Generally, complex dynamic interactions between clonogenic cancer cells and their supporting microenvironment form optimal conditions for tumor growth and invasion, using processes that are part of normal tissue development, repair and regeneration e.g. tissue remodeling.[11;12] Thus, tumor heterogeneity is caused by both cancer subclones and stromal cells. The most abundant glioblastoma stromal cells are vessel-associated cells (endothelial cells, pericytes, vascular smooth muscle cells and astrocytes), immune cells (macrophages, microglia and lymphocytes), neurons and oligodendrocytes. However, the distribution of these cells and the morphology of the extracellular matrix (e.g. necrosis) vary profoundly between tumors (inter-tumor heterogeneity) and within the same tumor (intra-tumor heterogeneity).[13;14]

1.4.2 Stepwise oncogenesis

Primary and secondary glioblastomas are indistinguishable under the microscope, but molecularly there are considerable differences. For example, mutation of the tumor suppressor gene *PTEN* (phosphatase and tensin homolog) and gene amplification of certain oncogenes - most commonly

the epidermal growth factor receptor gene *EGFR* - are hallmark alterations in primary glioblastoma, while isocitrate dehydrogenase 1 (*IDH1*), *TP53* and *ATRX* are frequently mutated in secondary glioblastoma. Therefore, the molecular pathways that lead to primary glioblastoma are clearly distinct from those giving rise to secondary glioblastoma (Figure 1).[15]

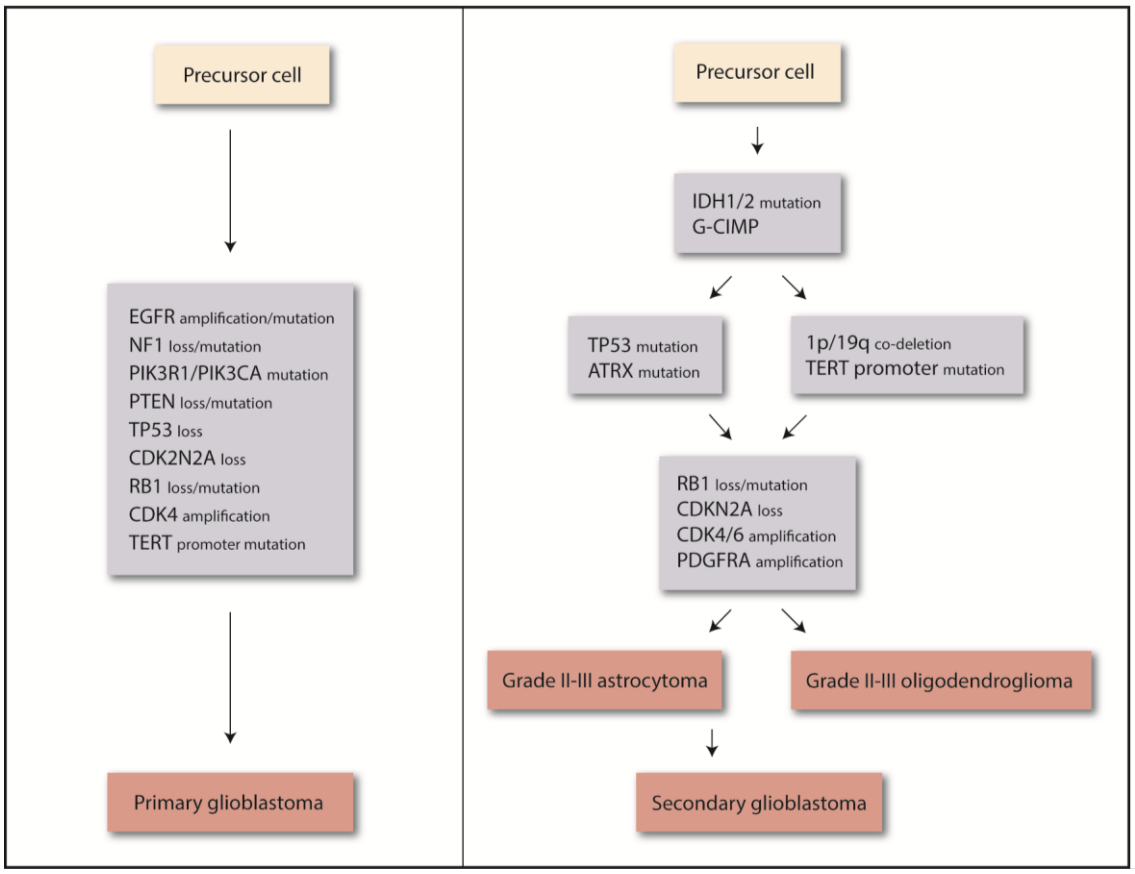


Figure 1. The genetic modifications leading to development of primary glioblastoma are markedly different from those leading to secondary glioblastoma. Modified from Aldape et al.[15]

1.4.3 Common signaling pathways

The majority of glioblastomas exhibit activation of the PI3K-AKT-mTOR and RAS-MAPK signaling pathways and these are therefore considered to be common oncogenic alterations in glioblastoma (Figure 2). In addition, the tumor suppressor retinoblastoma and p53 pathways are often disrupted by mutations in glioblastoma.[16] These four pathways are involved in multiple cancer hallmarks, including stimulation of cancer cell survival and proliferation.[17;18]

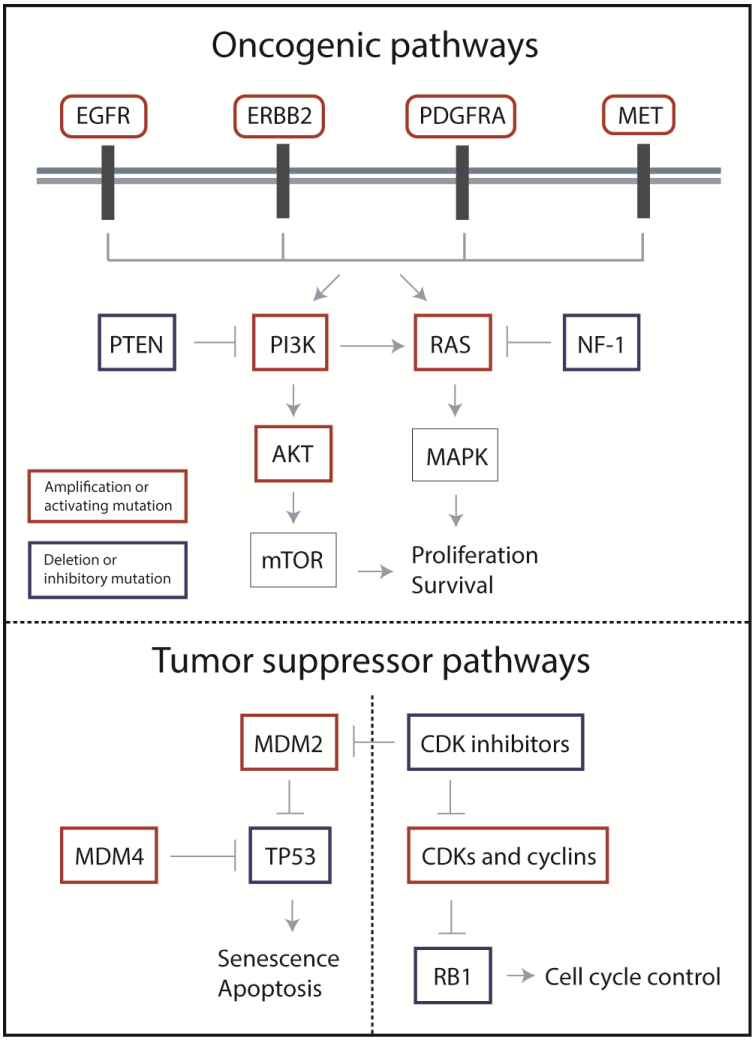


Figure 2. Common signaling pathways in glioblastoma. The oncogenic pathways, PI3K-AKT-mTOR and RAS-MAPK, are driven by activating alterations (red) in receptor tyrosine kinases and inhibiting alterations (blue) of NF1 and PTEN. Other alterations lead to inhibition of the tumor suppressor p53 and retinoblastoma pathways. Modified from Brennan et al.[16]

1.4.4 Molecular subtypes

Gene expression profiling using high-throughput genomic platforms has revealed transcriptional subtypes of glioblastoma. The first subtype classification system was established by Phillips et al, examining gene expression profiles of WHO grade III and IV gliomas. Three glioblastoma subtypes were identified and were termed proneural, mesenchymal and proliferative.[19] More recently, the initial The Cancer Genome Atlas Research Network (TCGA) expression profiling report by Verhaak et al, characterized four subtypes: proneural, neural, classical and mesenchymal.[20] When comparing these two classification systems, the two major subclasses, proneural and

mesenchymal, appear to be reproducibly defined while the other subtypes are less consistent.[21] As shown in Figure 3, these two subtypes also differ in genomic aberrations, as the proneural tumors are enriched with mutations in *IDH1* or amplification of the platelet-derived growth factor alpha gene (*PDGFRA*), and neurofibromin 1 (*NF1*) mutations are overrepresented in mesenchymal tumors. In addition, these subtypes apparently differ in relation to the common signaling pathways in glioblastoma, as the PI3K-AKT-mTOR pathway is more active in the proneural subtype and the RAS-MAPK pathway is more active in the mesenchymal subtype.[16;20] Accordingly, the molecular subtypes might reflect important glioblastoma biology which may be of relevance in the future clinical setting.

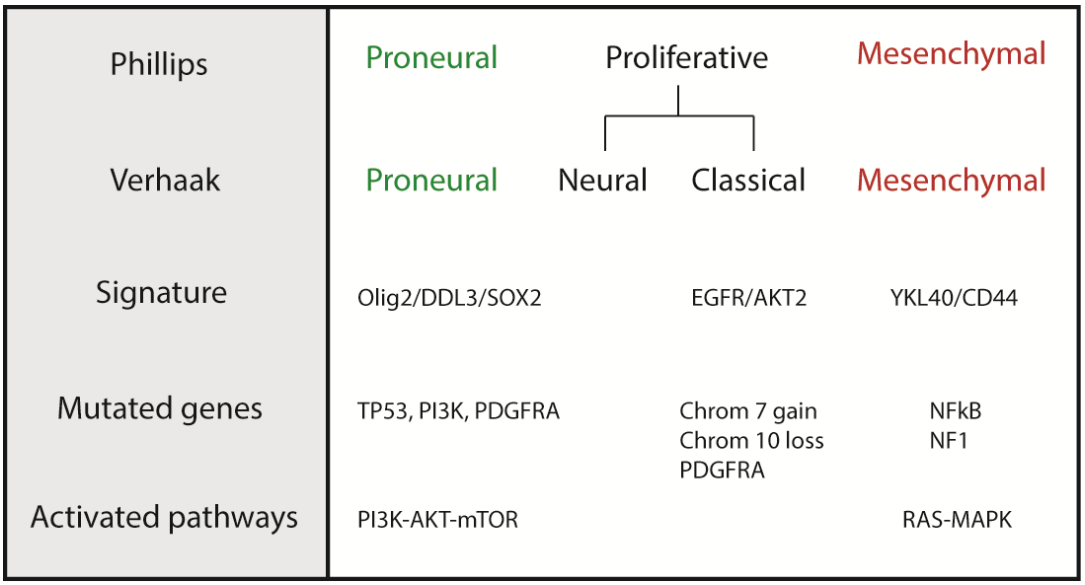


Figure 3. Molecular subtypes of glioblastoma. The proneural and mesenchymal transcriptional subtypes are the most consistent when comparing the Phillips and Verhaak classification. Differences in gene expression (signature), mutations and activated oncogenic pathways are shown according to subtypes. Modified from Aldape et al. [15]

Currently, the molecular characterization is mainly based on analysis of primary glioblastoma, and the molecular features of primary and recurrent glioblastoma may differ substantially. (Campos, Oncogene, 2016, *in press*) In this context, it has been shown that glioblastomas treated with cytotoxic agents shift towards a mesenchymal subtype at time of recurrence.[19]

1.5 Angiogenesis in glioblastoma

Multiple angiogenic factors are implicated in blood vessel formation. The most prominent pro-angiogenic factor, vascular endothelial growth factor A (VEGF), is overexpressed in glioblastoma compared to lower grades of glioma.[22] VEGF is mainly produced by tumor cells as a result of hypoxia, glucose deprivation, oxidative and mechanical stress, growth factors and mutations.[23] VEGF binds to vascular endothelial growth factor receptor 2 (VEGFR-2) on endothelial cells via a paracrine loop to increase permeability and promote angiogenesis by stimulating endothelial cell proliferation, migration and anti-apoptosis.[24] Accordingly, VEGF induces and maintains pathological angiogenesis which is associated with more aggressive tumor growth.

1.6 Anti-angiogenic therapy

Clinical strategies to inhibit tumor angiogenesis have mainly focused on targeting VEGF or VEGFR-2 with antibodies or small-molecule tyrosine kinase inhibitors.

Bevacizumab, a humanized monoclonal antibody targeting VEGF, is the most thoroughly studied anti-angiogenic agent in glioblastoma. Bevacizumab inhibits angiogenesis by specifically clearing circulating VEGF and hereby preventing activation of its receptors on endothelial cells. Consequently, bevacizumab does not have to cross the blood-brain barrier in order to be active. The large size of bevacizumab precludes oral administration and the terminal half-time of bevacizumab is 17-21 days.[25] This obligates patients to intra-venous administration every 2-3 weeks depending on treatment protocol.

Another anti-VEGF strategy has been the use of orally bioavailable VEGFR tyrosine kinase inhibitors. However, these agents are not as specific as antibodies and frequently impact other kinases. The most studied in glioblastoma is cediranib.[26]

1.7 Mechanisms of action of anti-angiogenic therapy

Due to extensive preclinical and clinical research our understanding of the mechanisms by which anti-angiogenic agents elicit tumor growth arrest has evolved.

The classical hypothesis proposed by Judah Folkman states that excessive pruning of blood vessels leads to increased hypoxia and nutrient deprivation, thereby limiting growth of the tumor or even causing regression.[27] This hypothesis has been widely accepted and confirmed in preclinical animal

studies.[28;29] However, the promising results from these studies have not been translated into the clinic.

More recently Rakesh K. Jain proposed a new hypothesis which suggested that anti-angiogenic therapy elicits its anti-tumor effect by transiently normalizing the abnormal tumor vasculature to increase tumor blood perfusion and improve both oxygen and drug delivery.[30] Evidence from several preclinical studies have confirmed this hypothesis.[31]

Other potential mechanisms of action of anti-angiogenic agents have been put forward, including intrinsic anti-tumor activity against glioblastoma stem-like cells,[32-34] reduction of vasogenic brain edema,[35] and reduction of VEGF-mediated immune suppression.[36]

The relative importance of these mechanisms of action regarding the therapeutic benefit of anti-angiogenic therapy is unknown. Recently, however, Batchelor et al provided clinical proof of concept that improved tumor blood perfusion, as a consequence of vascular normalization, was related to improved survival in glioblastoma patients treated with cediranib combinatory treatment.[37;38]

1.8 Vascular normalization theory

As illustrated in Figure 4, the formation and proliferation of tumor vasculature is a tightly regulated process kept in check by a balance of pro- and anti-angiogenic factors. However, in glioblastoma the balance is disrupted and tipped towards pro-angiogenic stimulation, resulting in pathological angiogenesis. These pathological blood vessels are disorganized, large in diameter, and have thickened basement membranes and decreased pericyte coverage. Functionally, this results in increased interstitial pressure and vascular permeability that can lead to edema, poor tumor blood perfusion and hypoxia. Hypoxia causes further increase in VEGF secretion, creating a vicious cycle for maintenance of angiogenesis and tumor progression. However, anti-angiogenic therapy can restore the balance between pro- and anti-angiogenic factors in the tumor, which in turn transiently normalizes tumor blood vessels and increases perfusion and improves oxygen and drug delivery. However, tipping the balance too far toward anti-angiogenesis may result in excessive vessel pruning, creating a hostile hypoxic tumor microenvironment, which may possibly fuel tumor progression.[31;39;40]

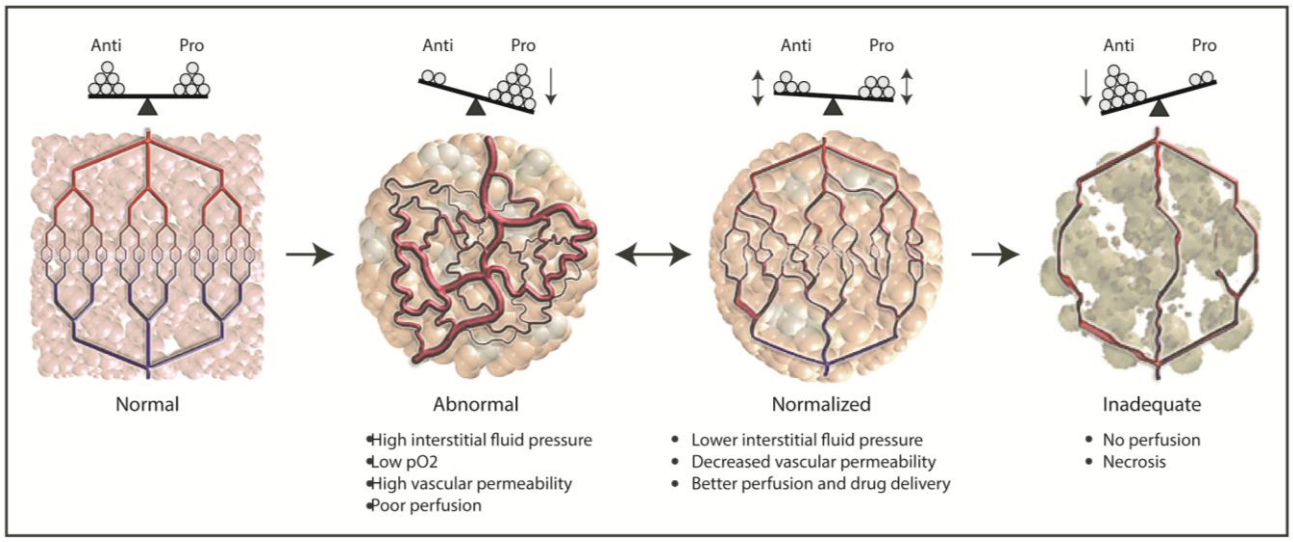


Figure 4. Vascular normalization theory. The disrupted balance between pro- and anti-angiogenic factors in glioblastoma can be restored by anti-angiogenic therapy, leading to a transient morphological and physiological normalization of blood vessels. Modified from Jain et al.[39]

1.9 Bevacizumab combination therapy in glioblastoma patients

The efficacy of bevacizumab combined with the topoisomerase I inhibitor irinotecan in glioblastoma was first described in 2005.[41] Today, bevacizumab is the most tested biological agent in glioblastoma.

Table 1 shows selected clinical trials investigating standard treatment with neoadjuvant bevacizumab and the randomized phase III trials of standard treatment with or without bevacizumab. These studies demonstrate that bevacizumab in combination with standard therapy prolongs PFS, but not OS. Furthermore, this was observed in the randomized phase-III GLARIUS trial of patients with non-methylated *MGMT* status who are known to have limited effect of standard therapy.

Table 2 summarizes trials selected trials with available response data on bevacizumab plus chemotherapy in the recurrent setting. Similarly to the first-line setting, bevacizumab has demonstrated impressive durable response rates, but no improvement in OS. This was recently confirmed in the randomized phase III BELOB trial (Table 2).

Currently, it is unclear whether bevacizumab influences quality of life positively in the total population of glioblastoma patients.[42-45]

Table 1. Bevacizumab combination regimens in first-line treatment

Design	*n*	Regimen	ORR (%)	PFS6 (%)	Median PFS (months)	Median OS (months)	Reference
		Neoadjuvant					
Phase-2 Randomized	31	**A**: Neoadjuvant IRI + BEV (8 weeks) Concomitant RT + IRI + BEV Adjuvant IRI + BEV	23	52	7.3	15.1	Hofland et al.[46]
	32	**B**: Neoadjuvant TMZ + BEV (8 weeks) Concomitant RT + TMZ + BEV Adjuvant TMZ + BEV	32	53	7.7	11.8	
Phase-2 Randomized	60	**A**: Neoadjuvant IRI + BEV (8 weeks) Concomitant RT + TMZ + BEV Adjuvant IRI + BEV	NA	62	7.1	11.1	Chauffert et al.[47]
	60	**B**: Concomitant RT + TMZ Adjuvant TMZ	NA	42	5.2		
Phase-2	41	Neoadjuvant TMZ + BEV (16 weeks) Concomitant RT + TMZ Adjuvant TMZ	24	NA	NA	11.7	Lou et al.[48]
		Concomitant and adjuvant					
Phase-3 Randomized 1:1	458	**A**: Concomitant RT + TMZ + BEV Adjuvant TMZ + BEV	38	NA	10.6	16.8	Chinot et al.[43] (AVAGlio)
	463	**B**: Concomitant RT + TMZ + placebo Adjuvant TMZ + placebo	18	NA	6.2	16.7	
Phase-3 Randomized 1:1	312	**A**: Concomitant RT + TMZ + BEV Adjuvant TMZ + BEV	NA	NA	10.7	15.7	Gilbert et al.[44] (RTOG 0825)
	309	**B**: Concomitant RT + TMZ + placebo Adjuvant TMZ + placebo	NA	NA	7.3	16.1	
Phase-3 Randomized 2:1 Non-*MGMT-meth.*	116	**A**: Concomitant RT + BEV + IRI Adjuvant BVZ + IRI	NA	79	9.7	16.6	Herrlinger et al.[45] (GLARIUS)
	54	**B**: Concomitant RT + TMZ Adjuvant TMZ	NA	43	6.0	17.5	

Abbreviations: ORR, overall response rate; PFS6, progression-free survival rate at 6 months; PFS, progression-free survival; OS, overall survival; BEV, Bevacizumab; IRI, Irinotecan; RT, radiotherapy; TMZ, temozolomide; NA, not available.

Selected neoadjuvant studies and phase III trials investigating bevacizumab containing regimens in first line treatment. Table modified and updated from Poulsen et al.[49]

Table 2. Bevacizumab and chemotherapy regimens in the recurrent setting

Design	*n*	First-line regimen	Regimen	ORR	PFS6	Median PFS (months)	Median OS (months)	Reference
Phase II	23	RT and/or chemo	BEV + IRI	61%	30%	4.6	9.2	Vredenburgh et al.[50]
Phase II	32	RT/TMZ+TMZ	BEV + IRI	25%	28%	5.2	7.9	Møller et al.[51]
Phase II	43	RT/TMZ+TMZ	BEV + IRI + cetuximab	34%	30%	3.7	6.7	Hasselbalch et al.[52]
Phase II	40	RT/TMZ+TMZ	BEV + IRI + carboplatin	33%	47%	5.9	8.3	Reardon et al.[53]
Phase II	85	RT/TMZ+TMZ	**A**:BEV	28%	43%	4.2	9.2	Friedman et al.[54]
Randomized								(BRAIN)
	82		**B**:BEV + IRI	38%	50%	5.6	8.7	
Phase II	50	RT/TMZ+TMZ	**A**: BEV	38%	16%	3	8	Taal et al.[55]
Randomized								(BELOB)
1:1:1	46		**B**: CCNU	5%	13%	2	8	
	44		**C**: BEV + CCNU	34%	42%	4	11	
Phase III	288	RT/TMZ+TMZ	**A**: BEV + CCNU	42%	NA	4.2	9.1	Wick et al.[42]
Randomized								(BELOB)
2:1	149		**B**: CCNU	14%		1.5	8.6	

Abbreviations: ORR, overall response rate; PFS6, progression-free survival rate at 6 months; PFS, progression-free survival; OS, overall survival; BEV, Bevacizumab; IRI, Irinotecan; RT, radiotherapy; TMZ, temozolomide; CCNU, lomustine; NA, not available.
Selected clinical trials with available response data on bevacizumab plus chemotherapy regimens. Data presented for glioblastoma patients only.

Taken together, bevacizumab in combination with standard therapy or second-line chemotherapy has failed to demonstrate a beneficial effect in the total population of glioblastoma patients. However, approximately 25-30% of patients achieve durable bevacizumab response and this group of patients has demonstrated improved survival as well as quality of life.[51;56;57] This highlights the importance of identifying predictive biomarkers for bevacizumab response.

The molecular subtypes have been suggested as candidate predictive factors for bevacizumab survival benefit in glioblastoma patients.[58-60] However, the results from these studies have been inconsistent. Recent data suggest that high plasma metalloproteinase 2 (MMP2) may be predictive for bevacizumab response.[61;62] Currently, no validated predictive tumor biomarkers for bevacizumab response have been identified.[40;63]

2. OBJECTIVES AND AIMS

The overall objective of the thesis was to identify predictive factors for bevacizumab response and resistance in recurrent glioblastoma patients. Accordingly, the specific aims were:

1. To investigate the predictive and prognostic impact of clinical and paraclinical factors in recurrent glioblastoma patients treated with bevacizumab and irinotecan therapy.

2. To analyze gene expression profiles of tumors in order to identify predictive biomarkers for bevacizumab response in recurrent glioblastoma patients treated with bevacizumab combination therapy.

3. To analyze transcriptional changes in paired glioblastoma samples before and after bevacizumab therapy in order to characterize possible response and resistance mechanisms.

3. RESULTS

The results section of this thesis comprises the following three studies:

1. **Urup T**, Dahlrot RH, Grunnet K, Christensen IJ, Michaelsen SR, Toft A, Larsen VA, Broholm H, Kosteljanetz M, Hansen S, Poulsen HS, and Lassen U. Development and validation of a prognostic model for recurrent glioblastoma patients treated with bevacizumab and irinotecan. *Acta Oncol*. 2016 Feb 1:1-5.

2. **Urup T**, Michaelsen SR, Olsen LR, Toft A, Christensen IJ, Grunnet K, Winther O, Broholm H, Kosteljanetz M, Issazadeh-Navikas S, Poulsen HS, and Lassen U: Angiotensinogen and HLA Class II Predict Bevacizumab Response in Recurrent Glioblastoma Patients. Submitted to Molecular Oncology for publication.

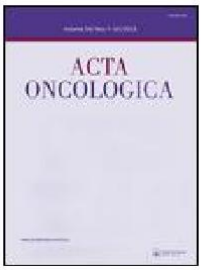
3. **Urup T**, Staunstrup LM, Michaelsen SR, Vitting-Seerup K, Bennedbæk M, Toft A, Olsen LR, Jønson L, Issazadeh-Navikas S, Broholm H, Hamerlik P, Poulsen HS, and Lassen U: Transcriptional changes induced by bevacizumab combination therapy in responding and non-responding recurrent glioblastoma patients. Submitted to International Journal of Cancer for publication.

3.1 Study I

Development and validation of a prognostic model for recurrent glioblastoma patients treated with bevacizumab and irinotecan

By

Thomas Urup, Rikke Hedegaard Dahlrot, Kirsten Grunnet, Ib Jarle Christensen, Signe Regner Michaelsen, Anders Toft, Vibeke Andrée Larsen, Helle Broholm, Michael Kosteljanetz, Steinbjørn Hansen, Hans Skovgaard Poulsen & Ulrik Lassen



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Development and validation of a prognostic model for recurrent glioblastoma patients treated with bevacizumab and irinotecan

Thomas Urup, Rikke Hedegaard Dahlrot, Kirsten Grunnet, Ib Jarle Christensen, Signe Regner Michaelsen, Anders Toft, Vibeke Andrée Larsen, Helle Broholm, Michael Kosteljanetz, Steinbjørn Hansen, Hans Skovgaard Poulsen & Ulrik Lassen

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ORIGINAL ARTICLE

Development and validation of a prognostic model for recurrent glioblastoma patients treated with bevacizumab and irinotecan

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ABSTRACT

Background Predictive markers and prognostic models are required in order to individualize treatment of recurrent glioblastoma (GBM) patients. Here, we sought to identify clinical factors able to predict response and survival in recurrent GBM patients treated with bevacizumab (BEV) and irinotecan.

Material and methods A total of 219 recurrent GBM patients treated with BEV plus irinotecan according to a previously published treatment protocol were included in the initial population. Prognostic models were generated by means of multivariate logistic and Cox regression analysis.

Results In multivariate analysis, corticosteroid use had a negative predictive impact on response at first evaluation (OR 0.45; 95% CI 0.22–0.93; $p = 0.03$) and at best response (OR 0.51; 95% CI 0.26–1.02; $p = 0.056$). Three significant ($p < 0.05$) prognostic factors associated with reduced progression-free survival and overall survival (OS) were identified. These factors were included in the final model for OS, namely corticosteroid use (HR 1.70; 95% CI 1.18–2.45; $p = 0.004$), neurocognitive deficit (HR 1.40; 95% CI 1.04–1.89; $p = 0.03$) and multifocal disease (HR 1.56; 95% CI 1.15–2.11; $p < 0.0001$). Based on these results a prognostic index able to calculate the probability for OS at 6 and 12 months for the individual patient was established. The predictive value of the model for OS was validated in a separate patient cohort of 85 patients.

Discussion and conclusion A prognostic model for OS was established and validated. This model can be used by physicians to risk stratify the individual patient and together with the patient decide whether to initiate BEV relapse treatment.

ARTICLE HISTORY
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Glioblastoma (GBM) is the most common primary malignant brain tumor in adults. Despite aggressive standard treatment, comprising maximal surgical resection, radiation therapy and concomitant and adjuvant temozolomide, newly diagnosed patients have a median overall survival (OS) of less than 15 months [1]. At tumor recurrence no standard treatment is available and most known options have limited clinical effect.

Several phase II studies of recurrent GBM patients treated with bevacizumab (BEV) combination therapy have shown high response rates [2,3] and patients who achieve response have an improved OS as well as quality of life [4–6]. Consequently, BEV combined with irinotecan (IRI) has been among the most frequently used treatments for recurrent GBM over the last years. Recently, the BELOB trial suggested that the combination of BEV with lomustine is superior to either agent used alone [7]. This is the first randomized study to indicate a survival benefit in recurrent GBM treated with BEV containing therapy. A confirmative phase III trial is awaited.

In non-BEV regimens used in recurrent GBM, the following prognostic factors have been associated with OS: Age [8], WHO performance status (PS) [8,9], corticosteroids [8,9], tumor size [9], multifocal disease [9], and frontal tumor location [9]. However, studies of these factors in recurrent GBM patients treated with a BEV containing therapy have shown inconsistent results [10–12]. More importantly, no validated predictive baseline markers associated with clinical durable response to BEV containing therapy have been identified.

The aim of this study was to identify prognostic and potentially predictive clinical baseline factors associated with response, progression-free survival (PFS) and OS in recurrent GBM patients treated with BEV plus IRI.

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Supplemental data for this article can be accessed here.

Materials and methods

Patients

This retrospective study was conducted in accordance with the Helsinki Declaration and was approved by the Danish Data Protection Agency (2006-41-6979).

Population 1 (initial cohort): By using a pharmacy database and a pathology database, we identified all patients with a pathologically confirmed diagnosis of GBM (WHO grade IV) who were treated at recurrence with BEV plus IRI between May 2005 and December 2013 at Rigshospitalet. During this period, BEV 10 mg/kg and IRI 125 mg/m^2 every two weeks could be prescribed to all recurrent GBM patients older than 18 years in WHO PS 0-2 at baseline. Patients had to have measurable progressive disease by contrast-enhanced magnetic resonance imaging (MRI) after standard therapy and be at least four weeks from prior chemotherapy and three months from completion of radiotherapy. For patients who had undergone relapse surgery a post-surgical MRI was performed prior to treatment initiation. Detailed inclusion and exclusion criteria are described in a previously published treatment protocol [5].

Population 2 (validation cohort): From a previously published cohort of 292 high-grade gliomas diagnosed between January 2005 and December 2009 at Odense University Hospital [13], we identified all patients with recurrent GBM treated with BEV and IRI.

Clinical follow-up

Clinical follow-up was performed every four weeks and MRI every eight weeks. MRI T1 and T2 sequences were employed, and in recent years the T2/fluid-attenuated inversion recovery (FLAIR) sequence was added. Treatment response and date of progression were evaluated (investigator assessment) using the Macdonald criteria [14]. In the more recent cases evaluated by the T2/FLAIR sequence, response and date of progression were retrospectively reviewed (by T.U., V.A.L.) based on Revised Assessment in Neuro-Oncology (RANO) criteria [15] and a high concordance with the initial evaluation was observed.

Statistical analysis

Response was estimated by employing logistic regression and the results presented by odds ratios with 95% confidence intervals (95% CI) and the area under the receiver operating characteristic curve. Survival probabilities were estimated with the Kaplan-Meier method. Multivariable analysis included covariates with p-value less than 10%. The Cox proportional hazards model was used for multivariable analysis. Assessment of the model assumptions was done using martingale residuals. The results are presented by hazard ratios with 95% CI and the concordance index (C-index) as a measure of discrimination [16]. A prognostic index for OS was developed based on the final multivariate analysis. The linear combination of the chosen covariates were assessed for 6- and 12-month survival using logistic regression and presenting the results by the sensitivity, specificity as well as the area under the receiver operating characteristic curve. The predictor for response, PFS and OS from the training set was calculated for the validation study and tested in this. *p*-values <0.05 were considered significant. Calculations have been performed using SPSS (v19.0, IBM Corp., Armonk, NY, USA), R (R Development Core Team, Vienna, Austria, //www.R-project.org) and SAS (v9.3, SAS institute, Cary, NC, USA) software.

Results

Patient characteristics

Population 1: A total of 219 recurrent GBM patients (73 women, 146 men) treated with BEV plus IRI were identified. Patient characteristics are summarized in Table I (full list according to screened factors, see Supplemental Table I, available online at //www.informahealthcare.com). After progression on BEV and IRI therapy, 22 patients underwent resurgery and 19 patients received various types of experimental treatments. Twenty patients were alive at the end of follow-up of whom seven had not progressed (median follow-up = 7.4 months, range 0.3–69 months).

Table I. Patient characteristics.

	Population 1 (n=219)	Population 2 (n = 85)
Gender, *n* (%)		
Female	73 (33)	33 (39)
Male	146 (67)	52 (61)
Age, years (range)		
Median	56 (21–79)	59 (27–79)
WHO performance status, *n* (%)		
0	78 (36)	18 (21)
1	98 (45)	36 (42)
2	40 (18)	28 (33)
3	0 (0)	3 (4)
Missing	3 (1)	0 (0)
Relapse surgery, *n* (%)		
Yes	111 (51)	28 (33)
No	108 (49)	57 (66)
Multifocal disease, *n* (%)		
Yes	72 (33)	16 (19)
No	142 (65)	69 (81)
Missing	5 (2)	0
Frontal location, *n* (%)		
Yes	52 (24)	32 (38)
No	167 (76)	53 (62)
Tumor size, mm^2 (range)[a]		
Median	1264 (90–8291)	NR
Use of corticosteroids, *n* (%)[b]		
Yes	155 (71)	55 (65)
No	59 (27)	30 (35)
Missing	5 (2)	0
Corticosteroid dose, mg (range)		
Median	37.5 (0–160)	25 (0–150)
Neurocognitive deficit, *n* (%)		
Yes	116 (53)	52 (61)
No	103 (47)	33 (39)
Response, *n* (%)		
Response (CR + PR)	66 (30)	40 (47)
Non-response (SD + PD)	131 (60)	45 (53)
Not evaluable	22 (10)	0 (0)
Median progression-free survival, months	5.0	5.9
Responders	9.1	8.0
Non-responders	4.1	3.4
Median overall survival, months	7.5	7.8
Responders	13.3	10.4
Non-responders	6.7	5.8

CR, complete response; NR, not registered; PD, progressive disease; PR, partial response; SD, stable disease.
[a]Sum of products of perpendicular diameters of all measurable enhancing lesions;
[b]Prednisolone >10 mg.

Population 2: A total of 85 patients were identified and included in the validation dataset. Characteristics are listed in Table I. Two patients were alive and all had progressed at the end of follow-up (median follow-up = 7.8 months, range 0.9–76 months).

Response, PFS and OS, Population 1

A response was found in 66 of 219 patients (30%; 95% CI 24–36). Twenty-two patients were non-evaluable at first response evaluation due to early progression, death or toxicity. Of the 197 evaluable patients, 66 patients were classified as responders (complete or partial response) and 131 patients as non-responders (stable or progressive disease). Among the responders 54 patients (82%) achieved response at first treatment evaluation. In univariate analysis, factors associated ($p < 10\%$) with a reduced chance of response at first response evaluation and at time of best response were: WHO PS 2 versus 0, corticosteroid use, higher corticosteroid dose and increasing tumor size.

Median PFS was 5.0 months (95% CI 4.2–5.3) and 6-month PFS was 33.5% (95% CI 27.3–39.8). In univariate analysis, factors associated with reduced PFS were: Increasing age ($p = 0.06$), WHO PS 2 vs. 0 ($p = 0.009$), multifocal disease ($p = 0.002$), corticosteroid use ($p = 0.01$), higher corticosteroid dose ($p = 0.005$), increasing tumor size ($p = 0.05$), neurological objective deficit ($p = 0.05$), neurocognitive deficit ($p = 0.02$), aphasia ($p = 0.01$), hemiparesis ($p = 0.03$) and ataxia ($p = 0.009$).

The median OS for all subjects was 7.5 months (95% CI 6.7–8.2). OS at 6 and 12 months were 63.8% (95% CI 57.4–70.2%) and 23.5% (95% CI 17.8–29.3%). Factors associated with decreased OS in univariate analysis were: WHO PS 1 versus 0 ($p = 0.01$), WHO PS 2 versus 0 ($p = 0.0005$), multifocal disease ($p < 0.00001$), corticosteroid use ($p = 0.0002$), higher corticosteroid dose ($p < 0.00001$), increasing tumor size ($p = 0.009$), neurological objective deficit ($p = 0.02$), neurocognitive deficit ($p = 0.01$), hemiparesis ($p = 0.02$), ataxia ($p = 0.02$) and aphasia ($p = 0.05$).

Multivariate analysis and prognostic models, Population 1

Table II summarizes the multivariate analyzes and prognostic models for response and survival endpoints. Corticosteroid dose (continuous) and corticosteroid use added equal predictability to the models for response, PFS and OS and in order to simplify the models it was decided to include only corticosteroid use. Use of corticosteroid was inversely related to response and the only predictor to have a significant impact on the likelihood of achieving a response at first evaluation (OR 0.45; 95% CI 0.22–0.93; $p = 0.03$) and at best response (OR 0.51; 95% CI 0.26–1.02; $p = 0.056$). Tumor size and WHO PS were not associated with response ($p > 0.20$). Nevertheless, it was decided to adjust the models for WHO PS along with the prognostic factors identified below. The model for response at first evaluation had a C-index of 0.66 and the model for best response had a C-index of 0.63.

Multivariate analysis of PFS identified neurocognitive deficit (HR 1.33; 95% CI 1.00–1.77; $p = 0.049$), multifocal disease (HR 1.56; 95% CI 1.15–2.11; $p = 0.004$) and corticosteroid use (HR 1.42; 95% CI 1.00–2.00; $p = 0.049$) as significant prognostic factors associated with an increased risk of progression. Remaining factors identified in univariate analysis were not significantly associated with PFS. However, it was decided to keep WHO PS in the model based on the reasons described above. The C-index for the model was 0.63.

Table II. Multivariate analysis of response at first evaluation, best response, progression-free survival and overall survival (Population 1).

	Response first evaluation OR (95% CI)	Best response OR (95% CI)	Progression-free survival HR (95% CI)	Overall survival HR (95% CI)
WHO PS, 1 vs. 0	1.15 (0.56–2.38) $p = 0.70$	1.18 (0.59–2.35) $p = 0.63$	1.01 (0.73–1.41) $p = 0.95$	1.16 (0.82–1.63) $p = 0.40$
WHO PS, 2 vs. 0	0.41 (0.10–1.65) $p = 0.21$	0.52 (0.16–1.70) $p = 0.28$	1.14 (0.73–1.78) $p = 0.58$	1.29 (0.82–2.03) $p = 0.27$
Neurocognitive deficit, yes vs. no	0.94 (0.48–1.85) $p = 0.86$	1.00 (0.53–1.90) $p = 0.99$	1.33 (1.00–1.77) $p = 0.049$	1.40 (1.04–1.89) $p = 0.03$
Multifocal disease, yes vs. no	0.62 (0.30–1.29) $p = 0.20$	0.67 (0.34–1.31) $p = 0.24$	1.56 (1.15–2.11) $p = 0.004$	1.87 (1.37–2.56) $p < 0.0001$
Corticosteroid use, yes vs. no	0.45 (0.22–0.93) $p = 0.03$	0.51 (0.26–1.02) $p = 0.056$	1.42 (1.00–2.00) $p = 0.049$	1.70 (1.18–2.45) $p = 0.004$
C-index	0.66	0.63	0.63	0.64

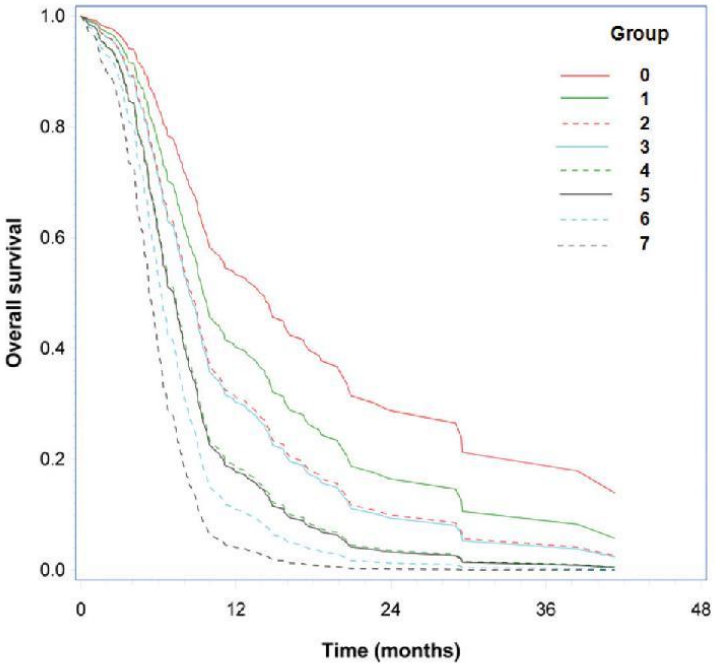
C-index, concordance index; WHO PS, WHO performance status.

Of factors identified by univariate analysis, three were found significantly associated with reduced OS by multivariate analysis when adjusted for WHO PS: neurocognitive deficit (HR 1.40; 95% CI 1.04–1.89; $p = 0.029$), multifocal disease (HR 1.87; 95% CI 1.37–2.56; $p < 0.0001$) and corticosteroid use (HR 1.70; 95% CI 1.18–2.45; $p = 0.004$). The C-index was 0.64.

Figure 1 shows the estimated survival curves based on the combinations of the three significant covariates included in the final model for OS. The median OS for the best group (negative for all three covariates) was 13.8 months and 5.3 months for patients in the group with the poorest signature (positive for all three covariates). The estimated 6- and 12-month OS corresponding to the eight possible combinations of the three markers are shown in Figure 1. The area under the curve (AUC) for 6-month survival was 0.67, with 51% sensitivity at 75% specificity and for 12-month survival, the AUC was 0.71 with 55% sensitivity at 75% specificity.

Validation of prognostic models

Application of the predictor established in Population 1 for best response to the validation set (Population 2) showed a non-significant association ($p = 0.22$) with a C-index of 0.58. The results of applying the predictor for PFS to the validation study showed a significant association ($p = 0.01$) with a C-index of 0.59. OS in the validation study demonstrated a significant association to the predictor estimated from the training set ($p = 0.03$) with a C-index of 0.62. Collectively, the predictor for response was not validated, while the model for PFS and OS was validated. The estimated OS probabilities for 6 and 12 months for the validation study using the predictor from the training set are shown in Figure 1.



Prognostic index				Population 1		Population 2	
Group	Neurocognitive deficit	Corticosteroid	Multifocal disease	OS6, % (95% CI)	OS12, % (95% CI)	OS6, % (95% CI)	OS12, % (95% CI)
0				84 (78-90)	53 (43-67)	76 (65-90)	44 (29-68)
1	X			77 (70-85)	40 (29-55)	73 (62-85)	38 (26-56)
2		X		72 (64-80)	31 (22-44)	72 (61-84)	36 (25-53)
3			X	71 (61-82)	30 (19-49)	70 (60-82)	34 (24-50)
4	X	X		62 (53-71)	19 (12-29)	67 (58-78)	30 (21-43)
5	X		X	61 (50-75)	18 (9-35)	66 (56-77)	28 (20-40)
6		X	X	53 (43-66)	11 (5-22)	65 (55-76)	26 (18-38)
7	X	X	X	40 (30-53)	4 (2-11)	60 (49-72)	20 (13-33)

Figure 1. Survival of risk groups. Survival curves (Population 1) and estimated survival probabilities at 6 and 12 months for Population 1 and 2. X indicates the presence of a prognostic factor. OS6, 6-month overall survival; OS12, 12-month overall survival.

Discussion

In this retrospective study of 219 patients with recurrent GBM treated with BEV plus IRI we found that prognostic factors were: Corticosteroid use, multifocal disease and neurocognitive deficit. Based on these factors a prognostic model for OS was established and validated in a cohort of 85 patients from another center.

The two cohorts included in this study differed in several patient characteristics. However, as median PFS and OS of 5.0 months and 7.5 months in the initial cohort were comparable to the validation cohort and other retrospective studies [10,12], we conclude that our cohorts are representative for the general population of recurrent GBM patients treated at different centers outside clinical trials.

In the initial cohort the response rate was 30% which was significantly lower than in the validation dataset. Response assessment is susceptible to considerable inter-observer variability and this may explain most of the difference in our observed response rates and why we were not able to predict

response in the validation cohort. In the initial cohort corticosteroid use was the only predictor inversely associated with response and prolonged PFS and OS. Corticosteroid use is a prognostic factor in recurrent GBM [8,9,11] and consequently may reflect a subgroup of patients less likely to achieve early benefit from BEV containing therapy in terms of response. In addition, corticosteroid dose changes are known to influence contrast enhancement, though the effect is considered transient (<2 weeks) [17]. Also worth considering is a possible corticosteroid-like effect of BEV influencing the contrast enhancement. Hypothetically, the use of corticosteroids at baseline may diminish the BEV-related effect on contrast enhancement at subsequent scans, reducing the likelihood of a radiological response. Lastly, use of corticosteroids was found significantly associated with a higher risk of developing severe (grade $\geq$3) lymphocytopenia. Whether this or other immunosuppressive effects of corticosteroids interfere with the efficacy of BEV remains unanswered.

In addition to corticosteroid use, neurocognitive deficit and multifocal disease were identified as independent poor prognostic factors for both PFS and OS. All three have previously been reported to affect survival in non-BEV-treated recurrent GBM patients [8,9,18]. In a recently published study on BEV-treated patients, corticosteroid use was associated with reduced PFS and OS [11]. To our knowledge, no other study has identified multifocal disease or neurocognitive deficit as being prognostic in BEV-treated recurrent GBM patients. In contrast to our expectations, and to previous studies [8,9,11,12], PS was not an independent prognostic factor. This might be due to a low number of patients with poor PS, which was highly associated with the use of corticosteroids. Consequently, the statistical power may not be adequate to detect an association between PS and survival endpoints.

In the present study we developed and validated prognostic models for GBM patients who receive BEV plus IRI at recurrence. Three risk factors predictive of early progression and mortality were identified: Neurocognitive deficit, corticosteroid use and multifocal disease. The prognostic model can help physicians to objectively inform patients about their prognosis and collaboratively decide whether the patient should or should not initiate BEV relapse treatment.

Declaration of interest

The authors have no conflicts of interest to declare.

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3.2 Study II

Angiotensinogen and HLA Class II Predict Bevacizumab Response in Recurrent Glioblastoma Patients

By

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Angiotensinogen and HLA Class II Predict Bevacizumab Response in Recurrent Glioblastoma Patients

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Keywords: Predictive model, angiotensin, vascular normalization, immune activation, anti-angiogenic treatment, glioblastoma, antigen presentation

Abstract

Background

Bevacizumab combination therapy is among the most frequently used treatments in recurrent glioblastoma and patients who achieve response to bevacizumab have improved survival as well as quality of life. Accordingly, the aim of this study was to identify predictive biomarkers for bevacizumab response in recurrent glioblastoma patients.

Methods

The study included a total of 82 recurrent glioblastoma patients treated with bevacizumab combination therapy whom were both response and biomarker evaluable. Gene expression of tumor tissue was analyzed by using a customized NanoString platform covering 800 genes. Candidate gene predictors associated with response were analyzed by multivariate logistic and Cox regression analysis.

Results

Two genes were independently associated with response: Low expression of angiotensinogen (2-fold decrease in *AGT*; OR=2.44; 95% CI: 1.45-4.17; P=0.0009) and high expression of a HLA class II gene (2-fold increase in *HLA-DQA1*; OR=1.22; 95% CI: 1.01-1.47; P=0.04). These two genes were included in a model that is able predict response to bevacizumab combination therapy in clinical practice. When stratified for a validated prognostic index, the predictive model for response was significantly associated with improved overall survival.

Conclusion

Two genes (low angiotensinogen and high HLA-class II expression) were predictive for bevacizumab response and were included in a predictive model for response. This model can be used in clinical practice to identify patients who will benefit from bevacizumab combination therapy.

Introduction

Glioblastoma is the most common primary malignant brain tumor in adults. Despite aggressive standard treatment, including maximal surgical resection and post-operative radiochemotherapy with temozolomide concomitantly and as maintenance, newly diagnosed patients have a median overall survival (OS) of less than 15 months.[1] At tumor recurrence no standard treatment is available and most known options have limited clinical effect.

Glioblastoma is characterized by increased angiogenesis and abnormal network of blood vessels. Anti-angiogenic agents inhibiting vascular endothelial growth factor A (VEGF) have been shown to normalize the tumor vasculature and improve blood flow, emphasizing the potential value of combining anti-angiogenic therapy with drugs targeting the tumor.[2;3] However, recent results from the first randomized phase III trial investigating chemotherapy with or without the VEGF-antibody bevacizumab did not demonstrate any difference in OS when considering the whole group of recurrent glioblastoma patients.[4] Still, approximately 30% of patients achieve durable bevacizumab response and this group of patients has demonstrated improved survival as well as quality of life.[5-7] This underscores the importance of identifying patients who will benefit from bevacizumab combination therapy. To date, no validated predictive tumor markers of a durable bevacizumab response have been identified. By analyzing gene expression profiles of glioblastoma patient tumors, the aim of this study was to identify predictive factors for bevacizumab response in recurrent glioblastoma patients.

Patients and Methods

Patients

All patients with pathologically confirmed glioblastoma (WHO grade IV) who were treated at recurrence with bevacizumab plus irinotecan between May 2005 and December 2011 at Rigshospitalet were assessed for eligibility. During this period, bevacizumab (10 mg/kg) and irinotecan (125 mg/m^2), administered every two weeks, could be prescribed to all recurrent glioblastoma patients in WHO performance status 0-2 according to a published treatment protocol.[8] Alternatively, both agents were combined with cetuximab in a phase 2 trial.[9] Eligibility criteria for this study were response evaluability and biomarker assessable tissue from the time of glioblastoma diagnosis. The criteria are specified in section 2.2-2.4 and a REMARK diagram is

shown in Supplementary Figure S1. The study was conducted in accordance with the Helsinki Declaration and was approved by the Danish Ethical Committee (H-2-2012-069).

Clinical Follow-up

According to the treatment protocol, patients had to have measurable progressive disease by contrast-enhanced MRI after standard therapy and be at least 4 weeks from prior chemotherapy and 3 months from completion of radiation therapy. For patients who had undergone relapse surgery a post-surgical MRI was performed prior to treatment initiation. Clinical follow-up was performed every 4-weeks and MRI every 8 weeks. Treatment response was evaluated based on the RANO criteria.[10] Patients were categorized according to their best response; patients who achieved complete response (CR) or partial response (PR) were classified as responders, while patients with stable disease (SD) or progressive disease (PD) were classified as non-responders. Patients not evaluable by MRI at first response evaluation (week 8) due to early toxicity, progression or death were classified as non-evaluable and excluded.

Sample acquisition and RNA preparation

A total of 90 archived formalin-fixed, paraffin-embedded tissue samples from time of initial glioblastoma diagnosis were collected and freshly cut sections (5 microns) were sent to HistogeneX, Belgium, and stored at 2-8°C. Tissue review was conducted by a pathologist blinded to identifiers and clinical outcome, and areas containing representative tumor cells were marked on hematoxylin and eosin-stained slides. Five samples with insufficient tumor tissue area for RNA analysis were excluded. Tumors were microdissected to enrich tumor cell RNA in the gene expression analyses. RNA was extracted using the High Pure RNA Paraffin Isolation kit (Roche, Ca. No. 03 270 289 001) and RNA extracts were stored at -80°C.

Gene Expression Data Generation

The platform consisted of 800 genes selected by Genentech using a custom code set for the NanoString gene expression platform (NanoString Technologies, Seattle, WA).[11] Genes were selected from the literature to allow glioblastoma molecular subtype classification according to Phillips' classifier,[12] and to cover genes regulating angiogenesis, immune system and other

glioblastoma-related cancer hallmarks. Analyses were performed using the software R version 3.1 (R Development Core Team, Vienna, Aurstria, http://www.R-project.org). Raw counts for 85 tumor samples were log2 transformed and normalized to 8 housekeeping genes recommended by Genentech and previously used on the AvaGlio dataset.[13] The normalization procedure is described in Supplementary Method 1. Based on the distribution of normalized counts, 3 outlier samples were identified and removed from further analysis, leaving 82 evaluable samples. Subtype labels were assigned to tumor samples by Genentech blinded to clinical outcome using the 31 gene classifier previously trained on the AVAglio dataset.[13]

Immunohistochemistry

Immunohistochemical analysis was conducted on 5 micron thick formalin-fixed, paraffin-embedded tissue sections. Following deparaffinization and protease treatment immunostaining was performed using the OptiView DAB IHC v4 Protocol (v1.00.0108) and the BenchMark ULTRA IHC staining Module (Ventana Medical System, Tucson, AZ, USA). The primary antibodies used were anti-HLA-DQA1 (dilution 1:150, Abcam, EPR7300), anti-HLA-DR (dilution 1:2000, DAKO, TAL 1B5) and anti-AGT (dilution 1:1500, LS Bioscience, LS-B6575).

Statistical analysis

Survival probabilities (PFS and OS) were estimated with the Kaplan-Meier method. Welch's test was performed to identify differentially expressed genes between groups. Treatment response was estimated by employing logistic regression (modelling the probability of response) and the results presented by odds ratios with 95% confidence intervals (95% CI) and the area under the receiver operating characteristic curve. The Cox proportional hazards model was used for modelling survival endpoints and results are presented as hazard ratios (HR) with 95% CI. Continues covariates were log transformed (log base 2) for analysis. Assessment of the model assumptions was done using Hosmer-Lemeshow test and martingale residuals. Five-fold cross-validation was applied to the analysis of response in order to assess the estimated model. Factors associated with response with *P*-values below 0.20 in univariate analysis were considered for multivariate analysis. Penalized maximum likelihood estimation was utilized for multivariate analysis and concordance indices (C-index) was calculated as a measure of discrimination.[14] *P*-

values < 0.05 were considered significant. Calculations were performed using SPSS (v19.0, IBM Corp., Armonk, NY), R version 3.1 and SAS (v9.3, SAS institute, Cary, NC).

Results

Patient characteristics

Of the 158 patients registered as receiving bevacizumab combination therapy at the time of relapse, 82 patients were response and biomarker evaluable (REMARK diagram, Supplementary Figure S1). Patient characteristics and clinical outcomes for the 82 patients are shown in Table 1. Response was observed in 29 patients (35%) of whom 22 (76%) achieved response at first treatment evaluation. After progression on bevacizumab combination treatment, 13 patients underwent surgical resection and 10 patients received various types of experimental treatments. Two patients were alive at the end of follow-up and all had progressed (median-follow-up: 8.3 months, range: 2-69 months).

Prognostic factors

Univariate analysis was performed to test if previously identified prognostic factors, shown in Table 1, were associated with response and to test if the cetuximab combined treatment had an impact on response. None of these factors were associated with response. In addition, we tested a recently established and validated prognostic index for recurrent glioblastoma patients treated with bevacizumab and irinotecan.[15] This index consists of 8 prognostic groups according to all possible combinations of the presence or absence of 3 independent prognostic factors: corticosteroid use (≥10mg Prednisolone), neurocognitive deficit (≥ minor) and multifocal disease. When applied to the current study cohort, the index was by univariate analysis significantly associated with PFS (P = 0.01) and OS (P = 0.005) but it was not associated with response (P = 0.45).

Molecular subtypes

Out of 82 samples, 27 were classified as proneural and 32 as the mesenchymal subtype. As illustrated in Supplementary Figure S2, the remaining 23 samples, categorized as proliferative or unclassified subtype, separated poorly from the proneural and mesenchymal subtypes.

Consequently, it was decided to analyze only the two robust subtypes as dichotomized variables: Proneural vs. non-proneural and mesenchymal vs. non-mesenchymal. By univariate analysis, shown in Supplementary Table S1, the two subtypes showed no association with response. Furthermore, no association with PFS or OS was observed in univariate analysis, nor when stratified for the prognostic index described above.

Identification of biomarkers associated with bevacizumab response

As shown in Figure 1, after pre-processing data, three steps were utilized to identify differentially expressed genes associated with treatment response. First, samples were divided into three groups according to best response: Response (CR + PR), stable disease (SD) and progressive disease (PD). To identify candidate genes differentially expressed between the two most extreme groups (response and PD) and to address unequal variance and unequal sample sizes of the groups, a Welch's t-test was performed (Step 2). Out of 792 genes, 9 genes were found significantly differentially expressed with a median fold change > 1.5 (Supplementary Table S2). These 9 genes were screened for association with response (CR + PR) versus non-response (SD + PD) by univariate analysis (Step 3). As shown in Supplementary Table S3, 5 genes were found associated with response ($P < 0.20$) and these were tested by multivariate analysis (Step 4, Supplementary Table S4). This analysis presented angiotensinogen (*AGT*) and a HLA class II gene (human leukocyte antigen complex class II DQ alpha 1, *HLA-DQA1*) as being the most interesting markers associated with response.

Predictors for response

Table 2 summarizes the final multivariate model for response. Indeed, low gene expression of angiotensinogen (2-fold decrease: OR=2.44; 95% CI: 1.45-4.17; $P = 0.0009$) and high expression of HLA class II (DQA1) (2-fold increase: OR=1.22; 95% CI: 1.01-1.47; $P = 0.04$) were significantly associated with an increased likelihood of response. None of the remaining gene candidates were significantly associated with response when added to the model. The final model for response had a high C-index of 0.78.

Association of predictors with PFS and OS

The two genes predictive for response were analyzed for association with PFS and OS. By univariate analysis, low gene expression of angiotensinogen was significantly associated with prolonged PFS (P = 0.01) and OS (P < 0.01), and high expression of HLA class II (DQA1) was significantly associated with prolonged OS (P = 0.03) but was not associated with PFS (P = 0.16). By multivariate analysis stratified for the prognostic index (Table 2), low expression of angiotensinogen was independently associated with prolonged PFS (2-fold decrease: HR=0.75; 95%CI: 0.59-0.94; P = 0.01) and OS (2-fold decrease: HR=0.70; 95%CI: 0.54-0.94; P = 0.005), while HLA class II (DQA1) expression did not significantly influence PFS or OS. The C-indices for the PFS and OS model were 0.67 and 0.68, respectively.

Clinical predictive model for response

In order to develop a model which in clinical practice can be used to predict bevacizumab response, the multivariate model for response was used to determine a cut point for angiotensinogen and HLA class II (DQA1) gene expression. Due to limitations and difficulties in response assessment, we prioritized a high specificity in preference to a high sensitivity in order to increase the likelihood of identifying patients not responding and not benefitting from bevacizumab treatment. Accordingly, a model able to predict bevacizumab response with a sensitivity of 66% at a specificity of 80% was established. In Figure 2, the linear curve is the gene expression threshold for angiotensinogen and HLA class II (DQA1) separating responders from patients not responding, illustrating that the gene expression threshold for each gene increases as a function of the other. In clinical practice this means that a patient with a relatively high expression of angiotensinogen (e.g. 900) is predicted to achieve response only if HLA class II (DQA1) is also relatively high (e.g. 1500), while another patient with the same expression of angiotensinogen but a lower expression of HLA class II (DQA1) will not respond to bevacizumab. The cross validation procedure confirmed the estimated model for response, both covariates were significant in all cases and the C-index was 0.75 for the test component.

When stratified according to the prognostic index, patients who according to the predictive model were predicted to respond had a borderline significantly longer PFS (P = 0.06) and significantly longer OS (P < 0.01) compared to patients predicted not to respond. This association with OS remained significant when patients progressing at the first response evaluation were excluded

from the analysis, indicating that the association of the model with OS is not due to including early progressors.

Immunohistochemistry

To examine the protein expression intensity and localization of angiotensinogen and HLA class-II proteins in glioblastoma, immunohistochemical analysis was performed on 10 tumor samples. These were the 5 showing the highest and the 5 showing the lowest gene expression levels of angiotensinogen on the NanoString platform. Staining for HLA-class II (DQA1) and HLA-DR as a control resulted in a similar granular cytoplasmic staining of macrophages and microglia located perivascular, around necrosis and diffusely in the stroma to a varying degree. There were no obvious differences in amount and location of HLA expressing cells across the samples with differing angiotensinogen expression (Supplementary Figure S3). As shown in Figure 3, angiotensinogen demonstrated a more diffuse staining in both reactive astrocytes, macrophages, microglia, glial tumor cells, endothelial cells and the extracellular matrix. The cellular staining was either cytoplasmic, nuclear or both. The intensity was varying between samples and intratumoral heterogeneity was most pronounced between malignant proliferating vessels and tumor cells. In glioblastomas with low gene expression, the staining intensity in tumor cells was mostly cytoplasmic and lower compared to tumor cells of glioblastomas with high angiotensinogen gene expression, which had a more pronounced staining in both cytoplasm and nucleus. The proliferating vessels in low angiotensinogen expressing glioblastomas seemed less compact, less fibrotic, and consisted of a mixture of positive and negative endothelial cells. In contrast, vessels in high angiotensinogen expressing tumors were more compact, fibrotic, proliferative and had smaller lumina.

Discussion

In this retrospective study of 82 recurrent glioblastoma patients treated with bevacizumab combination therapy, gene expression profiles of tumor tissue from the initial glioblastoma diagnosis were analyzed with the aim of identifying predictive factors for bevacizumab response. By analyzing candidate genes differentially expressed between responders and patients with early progressive disease, the expression of two genes were found independently associated with a

favorable response to bevacizumab therapy: These were low gene expression of angiotensinogen (*AGT*) and high gene expression of HLA class II (*HLA-DQA1*). Both were included in a clinically relevant model that can predict whether a patient is likely or not to respond to bevacizumab combination therapy.

In support of our findings, angiotensinogen has previously been found overexpressed in tumors of metastatic colorectal cancer patients not responding to bevacizumab combination therapy.[16] In addition, it has been shown that angiotensinogen and all components of the renin-angiotensin system, including the main effector peptide angiotensin-II, are expressed in glioblastomas.[17] The renin-angiotensin system appears to exert dual effects on the vasculature, as angiotensinogen has demonstrated anti-angiogenic signaling,[18] while angiotensin-II has been observed to induce angiogenesis.[19;20] Here we found that increasing angiotensinogen expression was associated with a higher level of vascular proliferation, suggesting an angiotensin-II dominating effect on the vasculature. Furthermore, high expression levels of angiotensinogen was associated with a more abnormal vessel architecture, characterized by excessive vascular remodeling and greater numbers of fibrotic blood vessels with reduced vessel lumina. These findings are also in line with angiotensin-II signaling which stimulates fibroblast-induced extracellular matrix remodeling and fibrosis.[21;22] Of interest, these angiotensin-II driven effects and in particular tumor fibrosis have been shown in preclinical models to compress blood vessels, while angiotensin-II inhibition reduced fibrosis and was able to decompress the vessels and increase tumor blood perfusion and drug delivery.[23;24] In contrast to these preclinical pancreatic and breast cancer models, which contain high frequencies of fibroblasts, patient-derived glioblastomas contain relatively small populations of fibroblasts located mainly within the vessels,[25-27] explaining the observed localization of fibrosis intravascularly. Accordingly, we hypothesize that locally produced angiotensinogen and angiotensin-II induce fibrosis within the vessels, hereby compressing blood vessel lumina, resulting in poorly perfused vessels, which cannot sufficiently be normalized by bevacizumab therapy. Interestingly, angiotensin-II inhibition has demonstrated a steroid-sparring and anti-edema effect in glioblastoma patients.[28] In addition, preclinical and retrospective studies suggest that combination of angiotensin-II inhibition and anti-angiogenic therapy at least has an additive effect.[29-31] Consequently, we are retrospectively investigating the efficacy and safety of this combination treatment in recurrent glioblastoma patients. This and other clinical studies,

including an ongoing phase III trial with angiotensin-II inhibition in combination with standard therapy (NCT01805453), will provide information on whether angiotensin-II inhibition should be administered to glioblastoma patients.

HLA-class II receptors are expressed on antigen presenting cells and by immunohistochemistry analysis expression was observed on microglia and macrophages. A possible explanation for the association of high HLA-class II gene expression and bevacizumab response is that HLA class II is up-regulated on local antigen presenting cells, which in turn directly activates and maintains a cytotoxic anti-tumor immune response. In such a scenario, bevacizumab treatment might induce an active immune response which is otherwise often reported to be skewed towards an immunosuppressive profile in glioblastoma.[32] Indeed, accumulating data indicate that anti-angiogenic agents activate anti-tumor immune cells and upon normalization of the vasculature increase the number of these tumor infiltrating immune cells.[33] Accordingly, HLA-class II expression may reflect an existing anti-tumor immune profile, which in concert with bevacizumab-induced immune activation may explain the association of HLA-class II with a beneficial effect of bevacizumab. Several clinical trials are currently evaluating combinatorial regimens of bevacizumab with different types of immunomodulating agents for glioblastoma patients.[34]

The molecular subtypes in our cohort had no impact on response, PFS or OS. Whether the proneural subtype (IDH1 wildtype) is a predictive factor for improved survival in bevacizumab treated glioblastoma patients, as suggested in the AvaGlio dataset,[13] remains elusive and has to be validated in a randomized trial. However, as subtype assignment has been shown to change following treatment and as a consequence of intratumoral heterogeneity, a clinically relevant subtype classification for recurrent glioblastoma has yet to be established.[12;35]

In summary, we identified low gene expression of angiotensinogen and high expression of a HLA-class II gene (*HLA-DQA1*) as independent predictors of bevacizumab response. Both genes are according to the literature involved in response and resistance mechanisms to anti-angiogenic combination therapies and we are currently testing these hypotheses pre-clinically as well as clinically. Based on the two identified genes we established a model which in clinical practice has the potential to predict bevacizumab response in recurrent glioblastoma patients. If validated, this model will contribute to identifying patients who will or will not benefit from bevacizumab combination therapy.

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Conflict of interests

The authors have no conflict of interests to declare.

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Tables and figures

Table 1. Patient characteristics	
	Total (*n* = 82)
Gender, *n* (%)	
Male	51 (62)
Female	31 (38)
Age, years (range)	
Median	56 (23-71)
WHO performance status, *n* (%)	
0	34 (42)
1	37 (45)
2	11 (13)
Prior lines of chemotherapy, *n* (%)	
1	73 (89)
2	9 (11)
Multifocal disease, *n* (%)	
Yes	21 (26)
No	61 (74)
Corticosteroid use, *n* (%)[a]	
Yes	61 (74)
No	21 (26)
Neurocognitive deficit, *n* (%)	
Yes	43 (52)
No	39 (48)
Bevacizumab combination therapy, *n* (%)	
Irinotecan	67 (82)
Irinotecan and cetuximab	15 (18)
Response, *n* (%)	
Response (CR+PR)	29 (35)
Stable disease	42 (51)
Progressive disease	11 (14)
Median progression-free survival, months	5.3
Responders	10.9
Non-responders	3.9
Median overall survival, months	8.2
Responders	13.8
Non-responders	7.5
Abbreviations: CR, complete response; PR, partial response [a]Prednisolone >10mg	

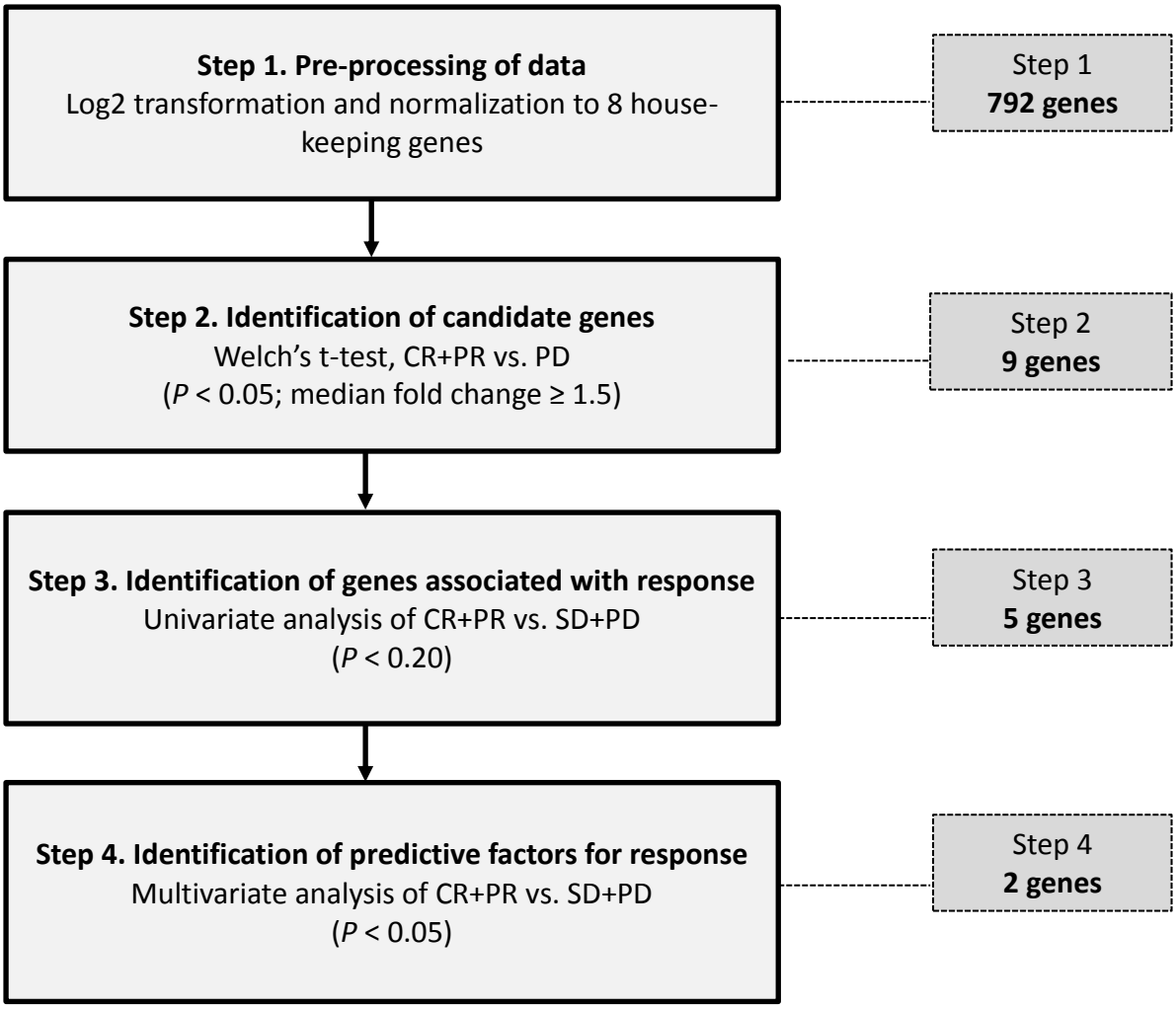

Figure 1. Flowchart for identification of differentially expressed genes associated with bevacizumab response. The number of genes shown in the right dotted box denotes the number of genes identified according to analytical steps.

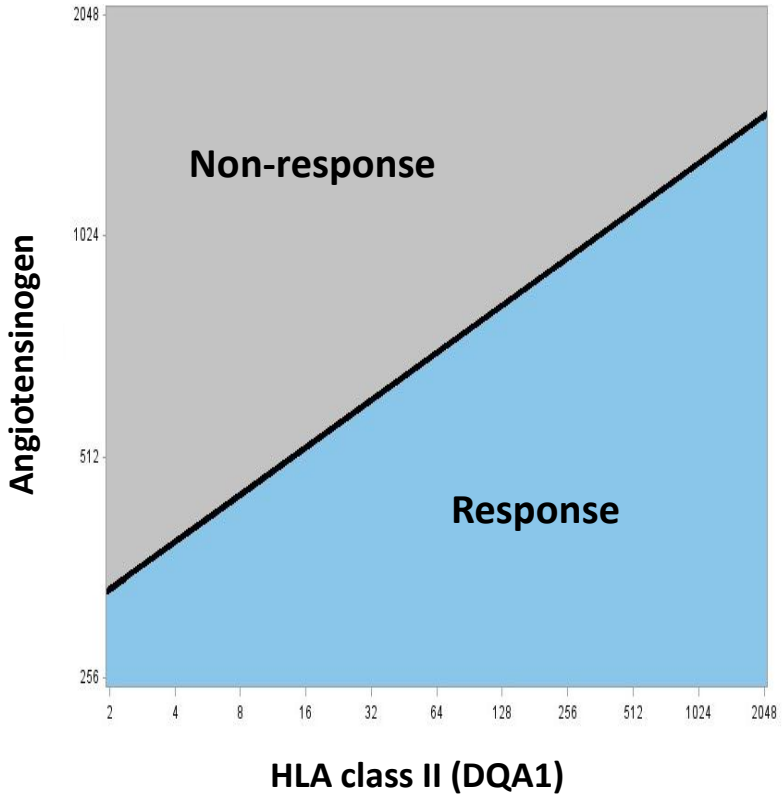

Figure 2. Predictive model for response to bevacizumab. The linear curve is the threshold for angiotensinogen and human leukocyte antigen complex class II DQ alpha 1 (DCA1) gene expression, separating responders from non-responders with a sensitivity of 66% and a specificity of 80%. X- and Y-axis represent gene expression count data normalized to reference genes.

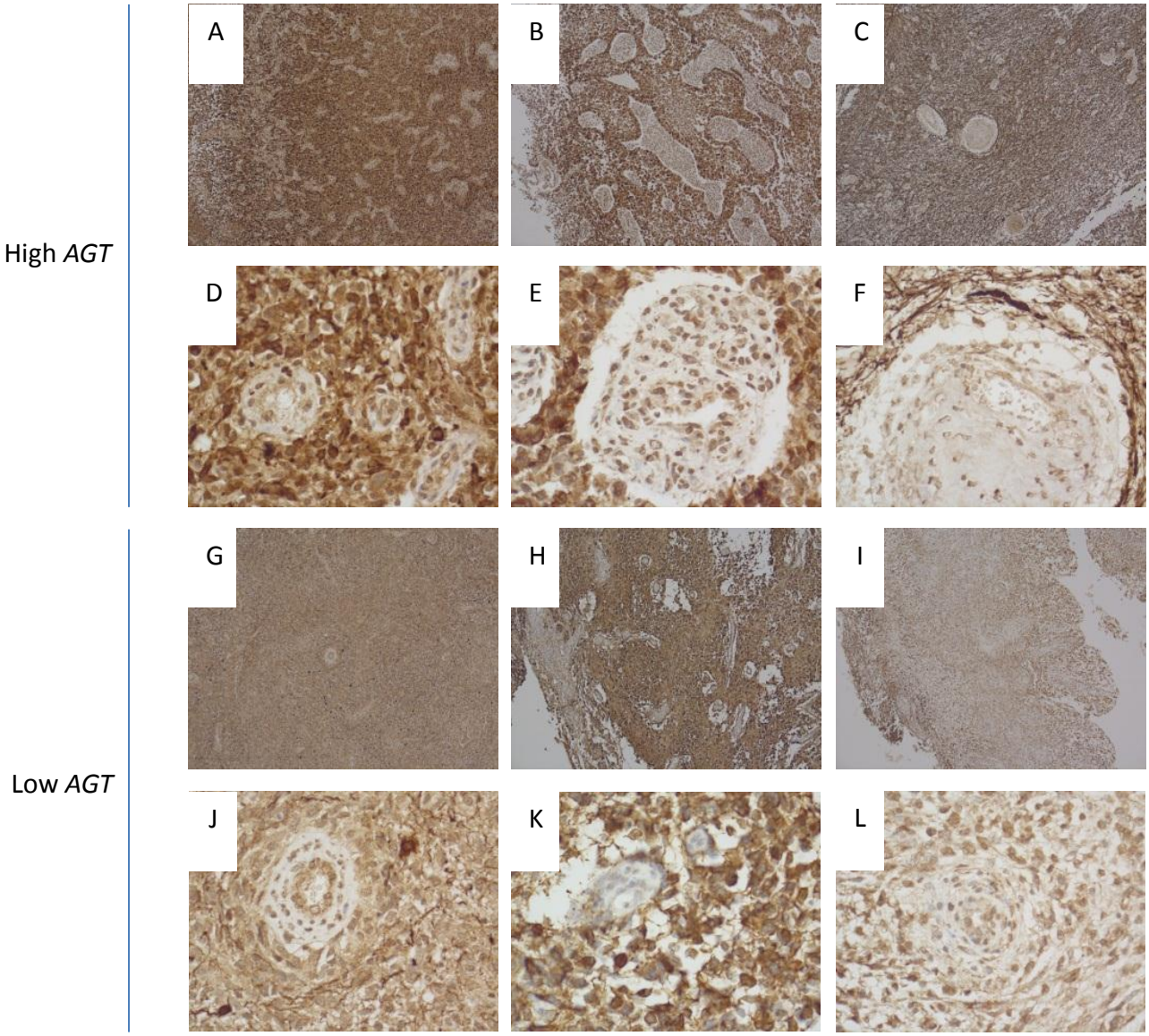

Figure 3. Immunohistochemistry for angiotensinogen. Overviews (×50) are shown for the 3 samples expressing the highest (A-C) and the lowest (G-I) angiotensinogen gene (*AGT*) expression. Tumor blood vessels (×400) of corresponding samples are shown below for high (D-F) and low (J-L) *AGT* expression.

Supplementary material

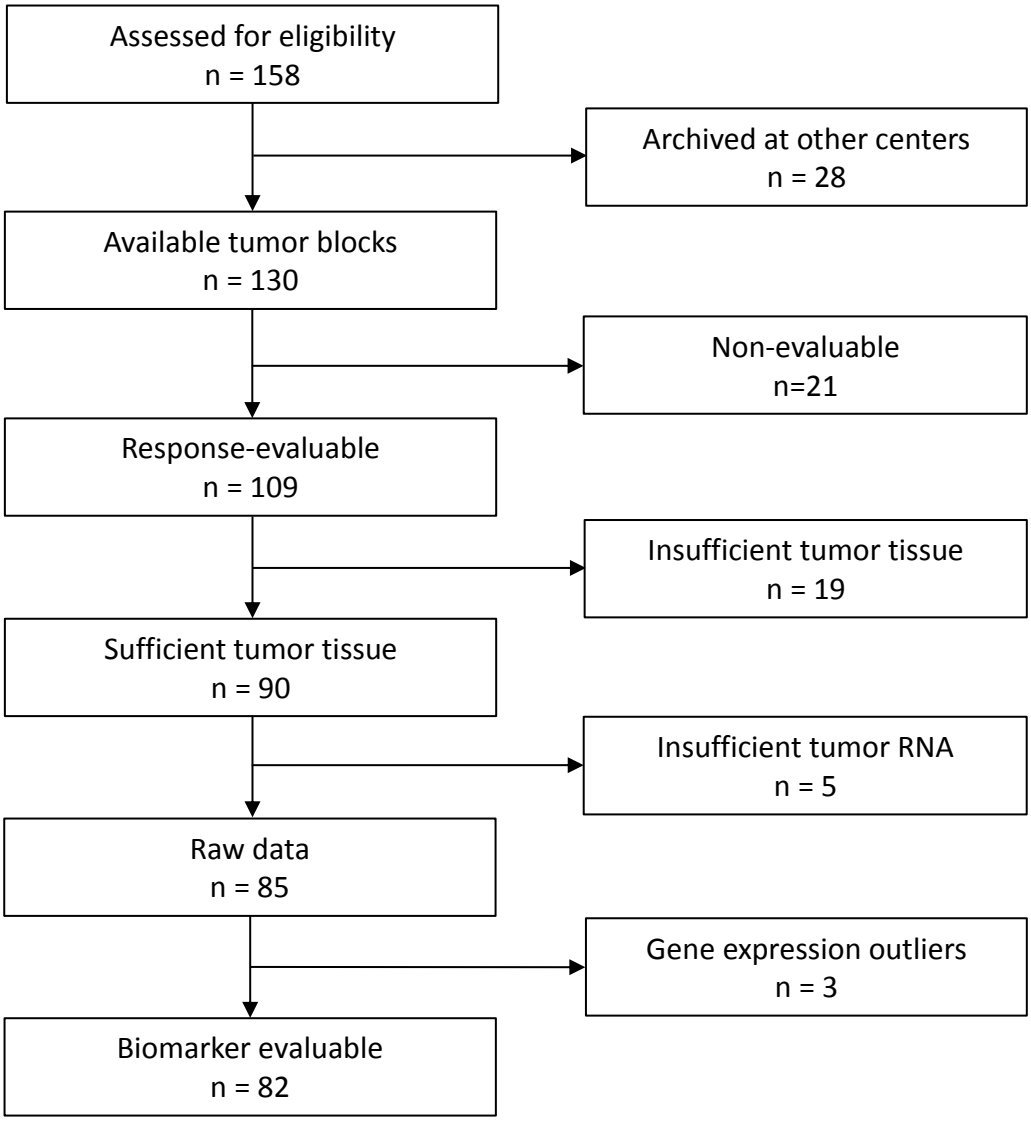

Figure S1. REMARK diagram for biomarker evaluable patients

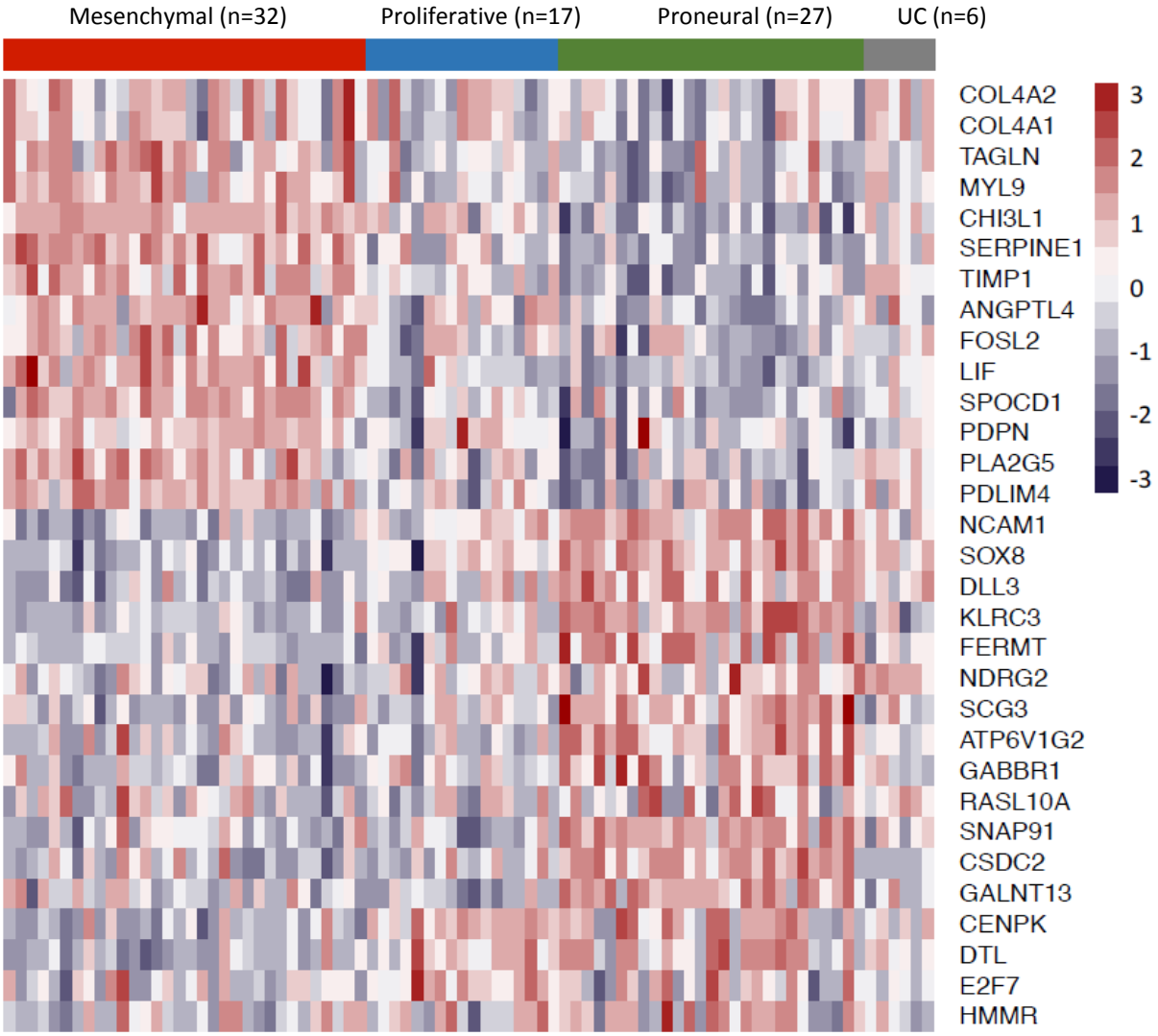

Figure S2. Heatmap of samples grouped by molecular subtypes as defined by Phillips et al. (columns) according to the 31 classifier genes (rows). Abbreviations: UC, unclassified.

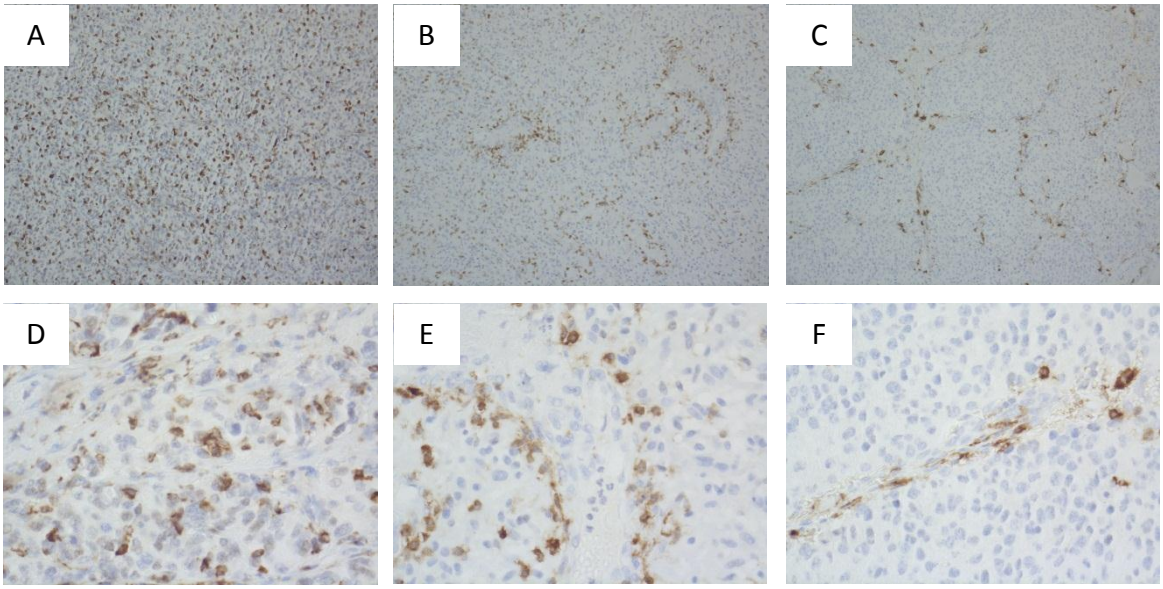

Figure S3. Immunohistochemistry for HLA-class II (hla-dqa1). Overviews (×100) are shown for samples with high (A-B) and low (C) angiotensinogen gene expression. Perivascular area (×400) of corresponding samples are shown below for high (D-E) and low (F) angiotensinogen expression.

Subtypes	Response		PFS		OS	
	OR (95%CI)	*P*-value	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
Proneural vs. non-proneural	0.89 (0.34-2.34)	0.81	1.06 (0.66-1.69)	0.81	0.93 (0.58-1.50)	0.78
Mesenchymal vs. non-mesenchymal	1.45 (0.58-3.63)	0.43	1.23 (0.79-1.93)	0.36	1.24 (0.79-1.97)	0.35
Non-proneural defined as mesenchymal, proliferative or unclassified samples. Non-mesenchymal defined as proneural, proliferative or unclassified samples.						

Suppl. Table S1. Univariate analysis of subtypes

	Median fold change	*P*-value
AGT	-2.6	0.005
BEST3	1.8	0.04
CDKN2B	1.5	0.03
E2F7	1.5	0.03
ENPP4	2.0	0.02
ERBB2	-2.0	0.049
HLA-DQA1	11.5	0.01
RTN1	1.5	0.005
TUSC3	1.8	0.003
Significant genes differentially expressed with a median fold change > 1.5 Minus indicates down-regulated genes in responders (CR+PR) compared to the progressive disease group (PD).		

Suppl. Table S2. Welch's t-test, comparing CR+PR vs. PD (Step 2)

	Response		
2-fold increase	OR (95% CI)	*P*-value	AUC
AGT	0.45 (0.27-0.75)	0.002	0.74
BEST3	1.11 (0.75-1.63)	0.61	0.52
CDKN2B	1.14 (0.88-1.49)	0.32	0.58
E2F7	0.93 (0.49-1.78)	0.83	0.54
ENPP4	1.41 (0.96-2.07)	0.08	0.63
ERBB2	0.61 (0.37-1.01)	0.05	0.61
HLA-DQA1	1.12 (0.95-1.33)	0.16	0.62
RTN1	1.23 (0.88-1.73)	0.22	0.57
TUSC3	1.30 (0.92-1.84)	0.14	0.61
Grey highlights selected candidate genes with a *P*-value < 0.20			

Table S3. Univariate analysis modelling the probability of response (Step 3)

	Response	
2-fold increase	OR (95%CI)	*P*-value
AGT	0.45 (0.26-0.79)	0.005
ENPP4	1.12 (0.71-1.77)	0.63
ERBB2	0.71 (0.41-1.25)	0.24
HLA-DQA1	1.17 (0.96-1.43)	0.11
TUSC3	1.04 (0.69-1.58)	0.84

Table S4. Multivariate analysis modelling the probability of response (Step 4)

Supplementary Methods

Log2-transformation and normalization of NanoString data

First, correction to positive controls was performed by multiplying the count for each gene in a given lane with a lane-specific scaling factor, calculated as the sum of all positive controls divided by the sum of positive controls for the given lane. Then, normalization to the reference genes *ACTB*, *AL-137727*, *GUSB*, *PPIA*, *RPLP0*, *TUBB*, *UBC*, and *VPS33B* was performed by multiplying each lane with a lane-specific normalization factor calculated as the mean of geometric means divided by the given lane's geometric mean. Lastly, lanes were corrected for technical noise by subtracting the sample background defined as the mean of the negative controls plus two times the standard deviation of the negative controls.

R-code is available at https://bitbucket.org/snippets/lronn/q89GM

3.3 Study III

Transcriptional changes induced by bevacizumab combination therapy in responding and non-responding recurrent glioblastoma patients

By

Thomas Urup, Line Mærsk Staunstrup, Signe Regner Michaelsen, Kristoffer Vitting-Seerup, Marc Bennedbæk, Anders Toft, Lars Rønn Olsen, Lars Jønson, Shohreh Issazadeh-Navikas, Helle Broholm, Petra Hamerlik, Hans Skovgaard Poulsen & Ulrik Lassen

Transcriptional changes induced by bevacizumab combination therapy in responding and non-responding recurrent glioblastoma patients

Authors

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Keywords: Anti-angiogenic, RNA-Seq, FFPE, reverse mesenchymal transition, TGF-beta

Abstract

Bevacizumab combined with chemotherapy is among the most frequently used treatments in recurrent glioblastoma, and patients who achieve response to bevacizumab have improved survival as well as quality of life. The aim of this study was to investigate transcriptional changes associated with response and resistance to bevacizumab therapy. Recurrent glioblastoma patients who had biomarker accessible tumor tissue surgically removed both before bevacizumab treatment and at time of progression were included. Patients were grouped into responders (n = 7) and non-responders (n = 14). Gene expression profiling of formalin-fixed paraffin-embedded tumor tissue was performed using RNA-sequencing. By comparing pretreatment samples of responders with those of non-responders no significant difference was observed. In a paired comparison analysis of pre- and posttreatment samples of non-responders 1 gene was significantly differentially expressed. In responders, this approach revealed 256 significantly differentially expressed genes of which 72 genes were down-regulated and 184 genes were up-regulated at the time of progression. Genes differentially expressed in responders revealed a shift towards a more proneural and less mesenchymal phenotype at the time of progression. These transcriptional changes were found associated to inhibition of TGF-β1 at the time of recurrence. In conclusion, bevacizumab combination treatment demonstrated a significant impact on the transcriptional changes in responders; but only minimal changes in non-responders. This suggests that non-responders progress due to intrinsic resistance while responders progress due to acquired resistance.

Introduction

Glioblastoma is the most common primary malignant brain tumor in adults. Despite aggressive standard treatment, including maximal surgical resection and radio-chemotherapy with concomitant and adjuvant temozolomide, newly diagnosed patients have a median overall survival of less than 15 months.[1] At tumor recurrence no standard treatment is available and most known options have limited clinical effect.[2]

Glioblastoma is characterized by excessive and aberrant angiogenesis. Anti-angiogenic agents inhibiting vascular endothelial growth factor A (VEGF) have been shown to normalize the tumor vasculature and improve blood flow and drug delivery.[3;4] This emphasizes the potential value of

combining anti-angiogenic therapy with drugs targeting the tumor. Bevacizumab, a VEGF targeting antibody, in combination with chemotherapy is among the most frequently used treatments in recurrent glioblastoma patients. Although this treatment regimen has not proved active in the total population of recurrent glioblastoma patients,[5] 25-30% of the patients achieve treatment response (defined as radiological and clinical improvement).[6] This group of patients has demonstrated improved survival as well as quality of life,[7-9] highlighting the importance of identifying predictive biomarkers for bevacizumab efficacy.

Glioblastoma consists of a mixture of cancer cell subclones, glial cells, stromal cells and immune cells, and each of these cell populations adds to the tumor heterogeneity. This complicates the interpretation of tumor biomarker analysis. Nevertheless, gene expression profiling of glioblastoma has revealed important findings, including preliminary evidence of survival benefit in distinct molecular subtypes treated with bevacizumab combination therapy.[10;11] However, the results of these studies have been inconsistent and we have along with others shown that the subtypes do not impact bevacizumab response in recurrent glioblastoma.[12] (Urup T., unpublished)

Due to the rarity of paired, biomarker evaluable, recurrent glioblastoma tissue samples, our current knowledge on bevacizumab response and resistance mechanisms is based on preclinical animal studies and small clinical case reports.[13-17] (Campos B, Oncogene, in press) Recently, novel gene expression technologies, including RNA-sequencing (RNA-Seq), have shown high performance on formalin-fixed paraffin embedded (FFPE) glioblastoma samples.[10;11] This will prove valuable for future clinical biomarker studies on archived tumor tissue.

In this study, we hypothesized that bevacizumab combination treatment exerts selective pressure on the tumors and creates adaptive transcriptional changes as tumors respond and progress. Accordingly, the aim was to identify transcriptional changes by RNA-Seq in tumor samples, before and after bevacizumab treatment in both responding and non-responding recurrent glioblastoma patients.

Patients and Methods

Patients

All patients with pathologically confirmed glioblastoma (WHO grade IV), who were treated at recurrence with bevacizumab plus irinotecan between May 2005 and December 2014 at

Rigshospitalet, were assessed for eligibility. Eligibility criteria for this study were 1) response evaluability and 2) biomarker accessible tumor tissue prior to bevacizumab treatment and at time of progression after bevacizumab treatment. The criteria are specified below. The study was conducted in accordance with the Helsinki Declaration and was approved by the Danish National Ethical Committee (H-2-2012-069).

Clinical Follow-up

Bevacizumab and irinotecan therapy was administered according to a published treatment protocol.[18] Prior to initiation of treatment the patients had to have measurable progressive disease by contrast-enhanced MRI after standard therapy and had to be at least 4 weeks from prior chemotherapy and 3 months from completion of radiation therapy. Post-surgical MRI was performed prior to treatment initiation. Clinical follow-up was performed every 4-weeks and MRI every 8 weeks. Treatment response was evaluated based on the RANO criteria.[19] Patients were categorized according to their best response; patients who achieved complete response (CR) or partial response (PR) were classified as responders, while patients with stable disease (SD) or progressive disease (PD) were classified as non-responders.

Sample acquisition and RNA preparation

A total of 264 patients were assessed for eligibility. Twenty-four response-evaluable patients had surgery before and after bevacizumab treatment and had archived paired FFPE tissue blocks at the Department of Pathology, Rigshospitalet. Tissue review was conducted by a neuropathologist, who was blinded to clinical outcome. The number of tumor cells was estimated based on hematoxylin and eosin-staining. Macrodissection was performed in a few cases to remove large amounts of normal brain tissue and only samples containing a tumor cell frequency > 50% were selected for RNA-extraction. If tumor blocks from relapse surgery prior to bevacizumab treatment were available and contained sufficient amount of tumor cells, they were included in preference to tumor blocks from time of glioblastoma diagnosis. All post-bevacizumab samples were obtained from relapse surgery following progression on bevacizumab treatment and no intermediate relapse therapy was administered. Three patients had an insufficient number of tumor cells in one of the paired tumor blocks and were excluded prior to analysis. Thus, a total of 21 patients with

paired tumor blocks were included in the study. Samples were sectioned into 3×10 micron thick FFPE sections and RNA was extracted from paired tumor blocks in three equal sample-sized batches using Deparaffinization Solution (Qiagen, Ca. No. 19093) and RNeasy FFPE kit (Qiagen, Ca. No. 73504). RNA extracts were stored at -80°C.

Library preparation for RNA-sequencing

Library preparation was carried out using the strand-specific Ovation Human FFPE RNA-Seq Library Systems from Nugen according to the instructions from the manufacturer. 250 ng of total RNA was used as input material for the cDNA synthesis and the double stranded cDNA was fragmented on the Covaris S2 (Covaris, Inc.) in microtubes using the following settings: duty cycle–10%/Intensity–5/cycles/burst-200 for a total of 180 seconds. RNA-Seq was performed on Illumina HiSeq 2500 (Illumina) as paired end sequencing 2x101 bases in Rapid Mode with 5 samples per run resulting in approximately 175 million paired-end reads per run. Raw data was converted to fastq files using CASAVA v1.8.2.

Filtering and differential expression analysis

Detailed description of the RNA-Seq analysis can be found in Supplementary Methods. Briefly, the raw sequencing data was trimmed with Trimmomatic and mapped to the human genome (*hg19*) with TopHat2.[20;21] Genes were annotated (Ensemble annotation, release 66) and expression levels were quantified using featureCounts.[22] Libraries with less than 10% of genes having more than 15 fragments were discarded (n = 6). Only genes with at least 10 fragments and an abundance of at least 3 Fragments Per Kilobase per Million reads (FPKM) in at least 5 libraries in any of the 4 patient-groups (Figure 1) were kept for further analysis (15,630 genes). Differential expression analysis was performed using edgeR (v. 3.12.0) either as paired analysis (comparison 1 and 3) or a batch-corrected analysis (comparison 2, see Figure 1). *P*-values were corrected for multiple testing using the False Discovery Rate (FDR) approach and genes with adjusted *P*-values < 0.05 were considered significant. Analysis was performed using the software R version 3.2.2 (R Development Core Team, Vienna, Aurstria, http://www.R-project.org). Expression data are available at the NCBI Geo datasets, accession number GSE79671.

Gene set enrichment analysis

Gene ontology gene-sets were downloaded (6th Jan 2016) from The European Bioinformatics Institute's official Gene Ontology mirror. Gene ontology terms from the 5th level of the hierarchical gene ontology term tree were used. Gene sets c2, c3, c6 and H were downloaded from The Molecular Signatures Database (MSigDB,[23] via http://bioinf.wehi.edu.au/software/MSigDB/). The enrichment analysis was done using a Fisher's exact test only considering the 15,630 tested genes, and *P*-values were FDR corrected and adjusted *P*-value < 0.05 were considered significant.

Ingenuity pathway analysis

Differentially expressed genes were analyzed by QIAGEN's Ingenuity Pathway Analysis (IPA) using the core analysis with default settings and 15,630 tested genes from the RNA-Seq dataset as background (IPA®,QIAGEN Redwood City, www.qiagen.com/ingenuity). The software uses a large database of curated data and computes a score for each network according to the fit of the set of genes supplied in the analysis. The scores were calculated by right-tailed Fisher's exact test. The scores derived from *P*-values, indicate the likelihood of supplied genes belonging to a network versus those obtained by chance. A consistency score (Z-score) > 2 or < -2 indicates with ≥ 99% confidence that a supplied gene network was not generated by chance alone. Enrichment of "canonical pathways" and "up-stream regulators" with a Z-score > 2 or < -2 were considered for analysis.[24]

Results

Patient characteristics

Patient characteristics and clinical outcome are shown in Table 1. All patients had received standard treatment with radio- and chemotherapy (temozolomide) prior to bevacizumab combination therapy. Half of the samples obtained prior to bevacizumab therapy were from surgery before standard treatment and the other half was from relapse surgery before bevacizumab initiation. Clinical characteristics did not differ significantly between responders and non-responders. Responders had a significantly longer progression-free survival compared to non-responders (*P* = 0.02) while no significant difference was observed in overall survival (*P* = 0.16).

Of the 42 samples, high quality RNA-Seq data was obtained on a total of 36 samples, leaving 20 “pre-bevacizumab samples” and 16 “post-bevacizumab samples” and 16 paired samples. Of the paired samples, 6 patients were classified as responders and 10 patients were classified as non-responders.

Group comparisons of gene expression profiles

To identify significantly differentially expressed genes between groups, comparison analyses were performed according to a pre-specified analytical strategy, shown in Figure 1. The comparison of pretreatment samples between responders (n = 7) and non-responders (n = 13) demonstrated no significantly differentially expressed genes. To identify transcriptional changes at the time of progression compared to before treatment a paired analysis was performed in non-responders (n = 10) and responders (n = 6), separately: In non-responders, 1 gene was significantly upregulated at the time of progression (Table S1). In responders, a total of 256 genes were found significantly differentially expressed, including 72 downregulated and 184 upregulated genes at the time of progression (Table S2 and S3). To analyze if the larger number of patients in the non-response group explained the absence of significant genes we performed a subsampling analysis. This analysis subsampled pairs of non-responders to random groups of 6 patients (100 times) and here we found that the mean number of differentially expressed genes (mean: 2.6; range: 0-33) was approximately 100 times lower than the number of significantly differentially expressed genes found in responders (Figure S1), indicating the results are not due to differences in sample sizes.
Collectively, we were not able to identify differentially expressed genes between pretreatment samples of responders and non-responders. Furthermore, bevacizumab combination therapy produced a significant impact on the transcriptional changes in responders at time of progression, but only minimal changes in non-responders.

Functional analysis of transcriptional changes in responders

In contrast to the non-protein coding gene (small nucleolar RNA, H/ACA box 22; *SNORA22*) identified in non-responders, several of the 256 genes identified in responders have been functionally well-characterized in published literature. To identify functional mechanisms related to the gene expression changes in responders, the Molecular Signatures Database (MSigDB) was

used to find gene ontologies and gene lists significantly enriched by the up- and down-regulated genes. The top-10 most significantly enriched gene ontologies and gene lists are shown in Table S4 and S5.

Gene ontology analysis showed that the up-regulated genes are implicated in nervous system development, neuron signaling and neuron differentiation. Down-regulated genes are involved in blood vessel development, collagen metabolism and endodermal differentiation.

Among the gene lists significantly enriched by the upregulated genes are three with high density of CpG-promoters bearing histone H3 trimethylation mark at K27 (H3K27me3). The gene list most significantly enriched by the down-regulated genes characterizes epithelial-mesenchymal transition. Interestingly, the mesenchymal and proneural subtypes defined by Verhaak overlapped significantly with the down-regulated and up-regulated genes, respectively.

Collectively, this analysis shows that responding glioblastomas when progressing express reduced levels of angiogenesis-related genes and higher levels of genes involved in neuronal development and signaling. Furthermore, the gene profiles changed towards a less mesenchymal phenotype and more proneural subtype at progression.

Dynamical changes in molecular subtype profiles

To investigate if bevacizumab treatment affects the expression of genes defining the molecular subtypes, gene expression in the paired samples of responders were analyzed according to subtype gene lists.[11;25] As shown in Figure 2, we observed that genes defining the Verhaak classical subtype were almost equally up- and downregulated, while the majority of mesenchymal genes were down-regulated at the time of progression. In contrast, most of the neural and proneural genes were upregulated at progression. According to the adapted Phillips classifier all genes of the mesenchymal subtype were down-regulated and all genes of the proneural subtype were up-regulated at progression.

Ingenuity pathway analysis of transcriptional changes in responders

In order to investigate the structure of possible regulatory networks underlying the significant gene expression changes in responders, we used the IPA. Unlike the enrichment analysis, IPA

allows identification of biological networks, including gene relationships and interactions, linked to specific known biological functions or pathways.

First, a canonical pathway analysis was performed to find activated or inhibited pathways. “Integrin signaling” was the only significantly inhibited pathway (Table S6). Fifteen canonical pathways were found activated and one of these involved “calcium signaling”, while the remaining 14 pathways all included protein kinase C related signaling genes (PRKCB, PRKCE, PRKCZ and others).

The IPA analysis identified 2 activated (estrogen receptor and SPDEF) and 4 inhibited (TGF-β1, SMAD3, ERK and ERRB2) upstream regulators (Table S7). Out of the 6 upstream regulators, TGF-β1 was the most significant (Z-score = -4.0) and *TGFB1* was the only gene which, based on our RNA-Seq data, trended toward a down-regulation in responders (raw *P* = 0.006; adjusted *P* = 0.15; log2 fold-change = -1.08) while this was not observed in non-responders (raw *P* = 0.57). Consequently, we focused on TGF-β1 and by using the mechanistic network function in IPA, we generated a plausible directional network from TGF-β1 and its closest related upstream regulator molecules. As shown in Figure 3, this network consisted of two inhibited regulators SMAD3, HIF1A and one activated regulator PPARG, of which *HIF1A* was the only gene trending toward a down-regulation in responders (raw *P* = 0.03; adjusted *P* = 0.36; log2 fold-change = -0.77), while this was not observed in non-responders (raw *P* = 0.51). These upstream-regulators directly or indirectly induce downstream effector molecules involved in cell-cycle check point regulation (CDKN1A) and extracellular matrix remodeling (SERPINE-1). These effector molecules in addition to others, shown in Supplementary Figure S3, were predominantly found transcriptionally down-regulated at the time of recurrence, suggesting that TGF-β1 signaling is inactive when tumors progress.

In summary, the pathway analysis showed that protein kinase C signaling was activated in progressing tumors. The analysis found TGF-β1 and HIF1A inhibited and a down-regulated trend was confirmed in the RNA-Seq data. These two up-stream regulators are known to regulate extracellular remodeling and cell-cycle, indicating that responding tumors at progression express reduced extracellular matrix remodeling and increased proliferation.

Histological changes

To investigate a possible association between the identified transcriptional changes and morphological changes of the tumor, we performed a non-blinded review of hematoxylin-eosin stained pre- and post-treatment samples. However, no gross differences or changes were observed in the amount and morphology of tumor cells, stromal cells, neural/glial cells, blood vessels or architecture of the extracellular matrix. Representative images of two responding glioblastomas are shown in Supplementary Figure S3.

Discussion

In this study of recurrent glioblastoma patients, we performed RNA-Seq on tumor tissue surgically removed before and after bevacizumab combination therapy. In line with others,[12] we found no significant differences between pretreatment samples of responders and non-responders. Considering the extreme inter-tumor heterogeneity of glioblastoma and the small sample size, this was not unexpected. Taking this into account, we performed paired analyses of before and after treatment samples. Our results reveal significant transcriptional changes in patients responding to bevacizumab while such changes were almost absent in patients not responding. This suggests that non-responding glioblastomas progress due to intrinsic resistance while bevacizumab sensitive tumors adaptively respond or progress to bevacizumab treatment.

By functional data mining of published literature, we studied the transcriptional changes of responding glioblastomas to uncover potential response and resistance mechanisms to bevacizumab treatment.

First, it is important to acknowledge that the patients underwent relapse surgery 2 months after bevacizumab treatment cessation. This may explain why no morphological changes in the vasculature were observed, as blood vessels can grow and remodel extensively within few weeks.[26] Nevertheless, gene ontology analysis found angiogenesis related genes significantly downregulated at the time of relapse. The reason for these conflicting findings remains unexplained.

The enrichment analysis revealed that up-regulated genes at time of progression were significantly overrepresented by genes involved in neural development and differentiation processes. Furthermore, up-regulated genes were significantly enriched by genes that are known to be up-

regulated due to de-methylation of H3K27 promoter regions - a process which is known to be related to decreased activity of Polycomp Repressive Complex 2 (PRC2) during differentiation.[27] Accordingly, epigenetic regulation may be associated with the up-regulated neural differentiation genes. However, no morphological changes were observed in regards to tumor or stromal cells.

It has previously been found that some glioma patients with recurrent disease after non-bevacizumab treatment shift from a proneural tumor into a mesenchymal subtype.[28] Preclinical glioblastoma studies have shown that adaptive resistance to anti-angiogenic agents is characterized by a transition to a mesenchymal phenotype.[16;17] In contrast, we observed that bevacizumab responding glioblastomas shift into a less mesenchymal and more proneural subtype when progressing.

By using the Ingenuity Pathway Analysis software, we identified TGF-β1 as the most central up-stream regulator associated with the identified gene expression changes. TGF-β1 was found inactivated at time of progression and this was associated with down-regulated extracellular matrix remodeling genes of which several define the mesenchymal subtype signature.[25] This suggests that the shift toward a less mesenchymal phenotype may be related to inactivation of TGF-β1 downstream signaling. In line with this finding, it has been shown in preclinical glioblastoma models that TGF-β signaling induces a mesenchymal shift, while inhibition of TGF-β prevents this shift.[29] Accordingly, the subtypes appear plastic and if the subtypes are representing specific cancer cell lineages, as originally proposed,[28] bevacizumab responding glioblastomas may transdifferentiate during progression. Another possibility is that interactions between tumor cells and microenvironment impact subtype classifications, similar to what is seen in epithelial cancers.[30] In this case, bevacizumab induced normalization of the tumor microenvironment and vasculature,[31;32] may change the gene profile accordingly.

Hypoxia has been identified as a central driver of acquired resistance to anti-angiogenic agents in preclinical animal models,[15] and hypoxia stimulates secretion of TGF-β which can lead to mesenchymal transition.[33] Thus, one could speculate that reduced hypoxia, as a consequence of bevacizumab-induced vascular normalization, may lead to TGF-β inhibition and reverse mesenchymal transition. Interestingly, and in line with our results, it has recently been found that breast cancer patients responding to bevacizumab demonstrate reduced levels of tumor hypoxia leading to reduced activity of TGF-β.[34]

The IPA analysis revealed a significant overrepresentation of genes associated with activated protein kinase C signaling at the time of relapse. This pathway has a central role in tumor-derived VEGF-induced angiogenesis, and in preclinical tumor models protein kinase C inhibitors have shown anti-angiogenic activity.[35] Accordingly, protein kinase C mediated VEGF secretion may induce resistance to bevacizumab and may serve as a target in bevacizumab responding glioblastoma patients.

To our knowledge this is the first study to demonstrate that bevacizumab combination treatment has a significant impact on transcriptional changes in a paired analysis of responding glioblastoma patients. Such changes were minimal in patients not responding. We observed that responding glioblastomas at the time of progression are characterized by a reverse mesenchymal transition, which may be related to bevacizumab-induced inhibition of TGF-β.

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Tables and figures

Table 1. Patient characteristics

	All patients (*n* = 21)	Responders (*n* = 7)	Non-responders (*n* = 14)
Gender, *n* (%)			
Male	11 (52)	5 (71)	6 (43)
Female	10 (48)	2 (29)	8 (57)
Age, years (range)			
Median	52 (21-70)	53 (35-65)	48 (21-70)
WHO performance status, *n* (%)			
0	10 (48)	2 (29)	8 (57)
1	9 (43)	4 (57)	5 (36)
2	2 (9)	1 (14)	1 (7)
Secondary glioblastoma, *n* (%)			
Yes	1 (5)	0	1 (7)
No	20 (95)	7 (100)	13 (93)
Standard glioblastoma therapy, *n* (%)			
Yes	20 (95)	7 (100)	13 (93)
No	1 (5)	0	1 (7)
Prior lines of chemotherapy, *n* (%)			
1	18 (86)	7 (100)	11 (79)
2	3 (14)	0	3 (21)
Tumor size, cm^2 (range)			
Median	9 (1-28)	11 (4-28)	8 (1-16)
Multifocal disease, *n* (%)			
Yes	2 (10)	0	2 (14)
No	19 (91)	7 (100)	12 (86)
Corticosteroid use, *n* (%)[a]			
Yes	14 (67)	3 (43)	11 (79)
No	7 (33)	4 (57)	3 (21)
Neurocognitive deficit, *n* (%)			
Yes	8 (38)	3 (43)	4 (29)
No	13 (62)	4 (57)	10 (71)
Primary sample, before bevacizumab, *n* (%)			
Initial glioblastoma diagnosis	10 (48)	3 (43)	7 (50)
Relapse surgery prior to bevacizumab	11 (52)	4 (57)	7 (50)
Time duration from relapse surgery (after bevacizumab), months			
to initiation of standard therapy, median	17	17	17
to last bevacizumab administration, median	2	2	2
Number of bevacizumab treatment cycles[b]			
Median	6	8	6

Bevacizumab combination therapy, *n* (%)			
Irinotecan	17 (81)	6 (86)	11 (79)
Irinotecan and cetuximab	4 (19)	1 (14)	3 (21)
Response, *n* (%)			
Response (CR+PR)	7 (33)	7 (100)	0
Stable disease	10 (48)	0	10 (71)
Progressive disease	4 (19)	0	4 (29)
Progression-free survival, months			
Median	5.4	10.8	3.9
Overall survival, months			
Median	10.8	14.3	8.6

Abbreviations: CR, complete response; PR, partial response.

[a] Prednisolone >10mg

[b] Two bevacizumab combination treatments (28 days) defined one treatment cycle

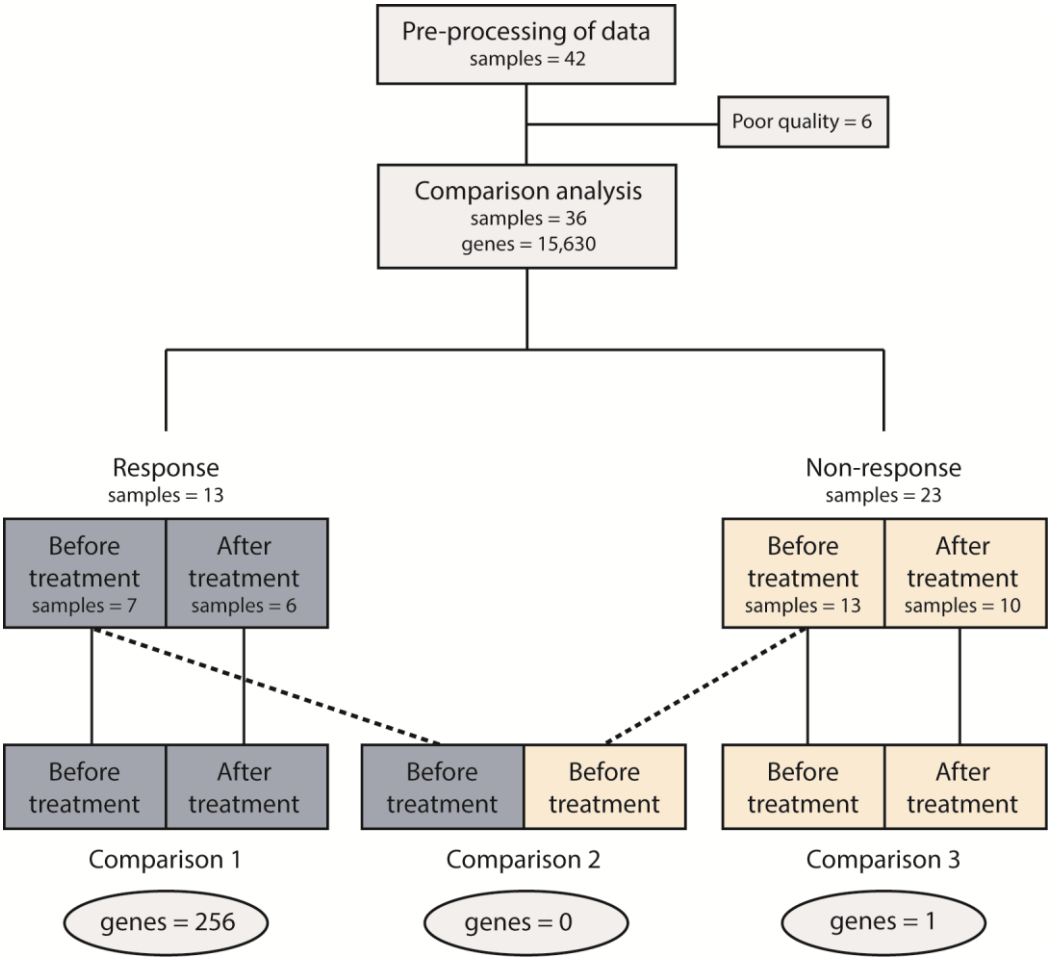

Figure 1. Flowchart for transcriptional comparison analysis. The number of genes identified significantly differentially expressed is shown below the three analyses.

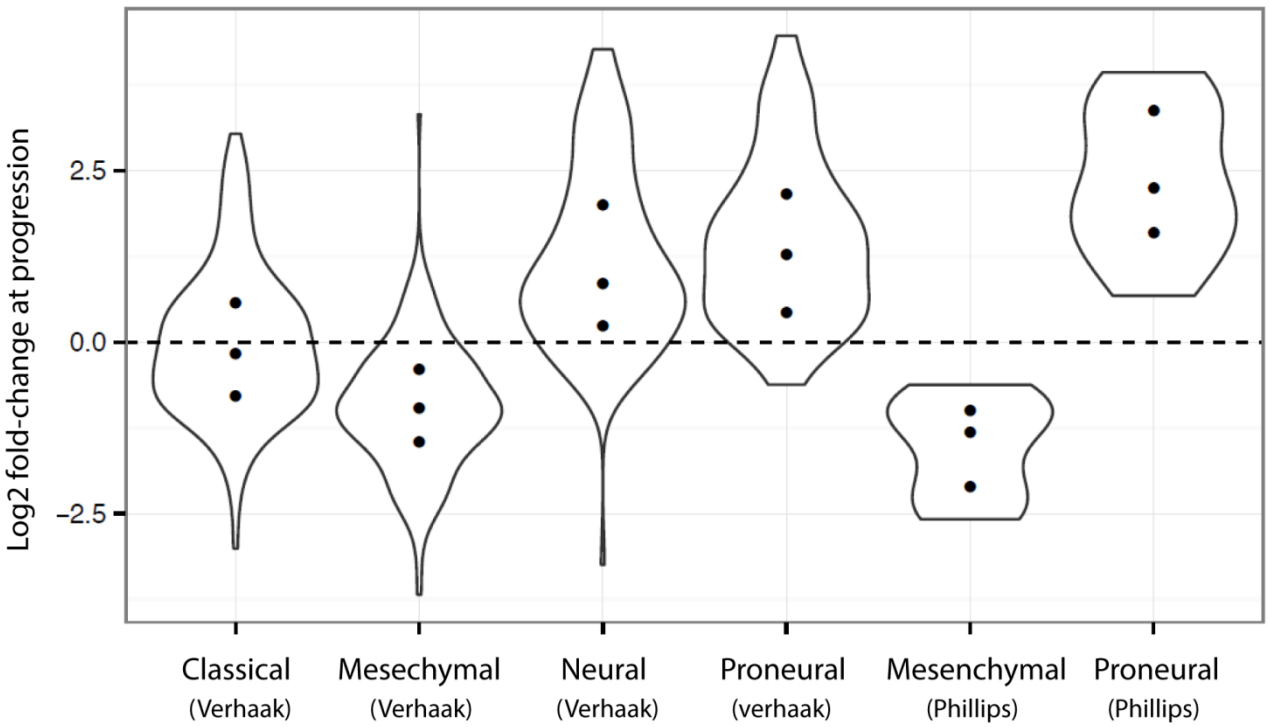

Figure 2. Paired gene expression fold-changes of genes defining molecular subtypes at the time of progression compared to before initiation of bevacizumab therapy in responding patients.
● indicates the gene expression change according to the 25% percentile of subtype genes.
* Modified Phillips classifier used on the AVAglio dataset.

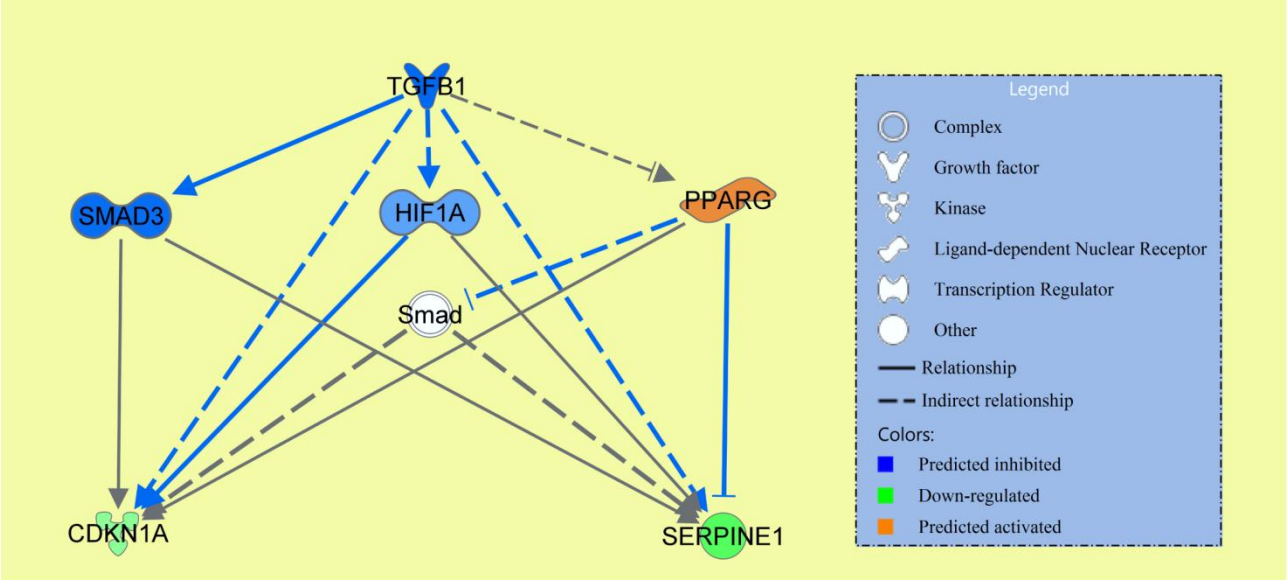

Figure 3. Mechanistic network of inhibited TGF-β1 generated on the basis of relationships to identified transcriptional changes in responders at the time of progression. The three closest related regulators in the network are SMAD3, HIF1A and PPARG and the two most related downstream molecules are CDKN1A and SERPINE1.

Supplementary Material

Table S1. Differentially expressed gene in non-responders (1 gene)

Gene ID	Gene Name	Adj. *P*-value	Log2 FC
ENSG00000206634	*SNORA22*	0.0004	1.68

Table S2. Down-regulated genes in responders (72 genes)

Gene ID	Gene Name	Adj. *P*-value	Log2 FC
ENSG00000168542	*COL3A1*	1.8E-07	-2.82
ENSG00000108821	*COL1A1*	3.1E-06	-3.01
ENSG00000106366	*SERPINE1*	6.6E-06	-2.59
ENSG00000163359	*COL6A3*	6.0E-05	-2.59
ENSG00000148848	*ADAM12*	0.0003	-2.44
ENSG00000130635	*COL5A1*	0.0005	-2.33
ENSG00000187498	*COL4A1*	0.0006	-2.47
ENSG00000164692	*COL1A2*	0.0006	-2.23
ENSG00000187678	*SPRY4*	0.0006	-2.18
ENSG00000110492	*MDK*	0.0011	-2.21
ENSG00000134871	*COL4A2*	0.0025	-2.12
ENSG00000137801	*THBS1*	0.0034	-1.99
ENSG00000091136	*LAMB1*	0.0040	-1.70
ENSG00000112769	*LAMA4*	0.0042	-1.67
ENSG00000107796	*ACTA2*	0.0051	-1.97
ENSG00000018280	*SLC11A1*	0.0065	-2.01
ENSG00000146674	*IGFBP3*	0.0071	-2.29
ENSG00000132530	*XAF1*	0.0071	-1.88
ENSG00000150636	*CCDC102B*	0.0089	-2.97
ENSG00000181104	*F2R*	0.0010	-1.73
ENSG00000010327	*STAB1*	0.0106	-1.45
ENSG00000115414	*FN1*	0.0108	-1.80
ENSG00000157227	*MMP14*	0.0113	-1.74
ENSG00000163430	*FSTL1*	0.0115	-1.37
ENSG00000261295	*RP11-524D16__A.3.1*	0.0129	-3.53
ENSG00000149948	*HMGA2*	0.0146	-2.28
ENSG00000174807	*CD248*	0.0146	-2.13
ENSG00000120318	*ARAP3*	0.0155	-2.10
ENSG00000226053	*RP5-1070A16.1.1*	0.0175	-5.83
ENSG00000103196	*CRISPLD2*	0.0175	-2.79
ENSG00000061337	*LZTS1*	0.0185	-1.87
ENSG00000120708	*TGFBI*	0.0187	-1.94
ENSG00000082074	*FYB*	0.0187	-1.86
ENSG00000163694	*RBM47*	0.0187	-1.74
ENSG00000141753	*IGFBP4*	0.0187	-1.60
ENSG00000161638	*ITGA5*	0.0197	-1.81
ENSG00000182718	*ANXA2*	0.0204	-1.37
ENSG00000184060	*ADAP2*	0.0221	-1.92
ENSG00000135424	*ITGA7*	0.0229	-1.65
ENSG00000159216	*RUNX1*	0.0230	-1.52
ENSG00000150551	*LYPD1*	0.0232	-2.22
ENSG00000196083	*IL1RAP*	0.0232	-1.96
ENSG00000111252	*SH2B3*	0.0232	-1.37
ENSG00000147614	*ATP6V0D2*	0.0233	-6.63
ENSG00000183486	*MX2*	0.0233	-3.03
ENSG00000124762	*CDKN1A*	0.0233	-1.71
ENSG00000186470	*BTN3A2*	0.0240	-2.07
ENSG00000142798	*HSPG2*	0.0240	-1.66
ENSG00000010610	*CD4*	0.0241	-1.67
ENSG00000177469	*PTRF*	0.0269	-1.36
ENSG00000076706	*MCAM*	0.0279	-1.47
ENSG00000106991	*ENG*	0.0287	-1.70
ENSG00000173068	*BNC2*	0.0316	-2.55
ENSG00000149257	*SERPINH1*	0.0321	-1.68
ENSG00000221818	*EBF2*	0.0326	-4.49
ENSG00000213949	*ITGA1*	0.0326	-2.01
ENSG00000134013	*LOXL2*	0.0333	-1.80
ENSG00000130429	*ARPC1B*	0.0350	-2.06
ENSG00000124813	*RUNX2*	0.0353	-1.75
ENSG00000173369	*C1QB*	0.0357	-1.47
ENSG00000182492	*BGN*	0.0367	-1.43
ENSG00000143226	*FCGR2A*	0.0392	-1.35
ENSG00000261468	*RP11-1024P17.1.1*	0.0426	-5.36
ENSG00000186407	*CD300E*	0.0427	-3.18
ENSG00000066336	*SPI1*	0.0433	-1.57
ENSG00000102265	*TIMP1*	0.0437	-1.80
ENSG00000130052	*STARD8*	0.0444	-2.80
ENSG00000060138	*CSDA*	0.0452	-1.51
ENSG00000137745	*MMP13*	0.0466	-5.95
ENSG00000100292	*HMOX1*	0.0466	-1.94
ENSG00000254369	*RP1-170O19.2.1*	0.0493	-6.55
ENSG00000128641	*MYO1B*	0.0496	-1.80

Table S3. Up-regulated genes in responders (184 genes)

Gene ID	Gene Name	Adj. *P*-value	Log2 FC
ENSG00000132692	*BCAN*	3.8E-05	3.66
ENSG00000175161	*CADM2*	4.5E-05	2.20
ENSG00000104888	*SLC17A7*	0.0004	6.35
ENSG00000183248	*AC010336.1*	0.0005	2.40
ENSG00000148798	*INA*	0.0006	6.04
ENSG00000175497	*DPP10*	0.0006	4.75
ENSG00000104833	*TUBB4A*	0.0006	4.12
ENSG00000112290	*WASF1*	0.0006	3.33
ENSG00000261786	*RP4-555D20.2.1*	0.0010	3.54
ENSG00000152932	*RAB3C*	0.0011	4.93
ENSG00000165443	*PHYHIPL*	0.0011	2.28
ENSG00000177511	*ST8SIA3*	0.0014	5.10
ENSG00000100167	*SEPT3*	0.0015	3.73
ENSG00000008086	*CDKL5*	0.0023	3.38
ENSG00000077279	*DCX*	0.0023	3.05
ENSG00000198910	*L1CAM*	0.0024	4.23
ENSG00000166897	*ELFN2*	0.0030	3.39
ENSG00000124507	*PACSIN1*	0.0040	5.23
ENSG00000008056	*SYN1*	0.0040	4.97
ENSG00000104435	*STMN2*	0.0040	4.71
ENSG00000132639	*SNAP25*	0.0040	3.98
ENSG00000107758	*PPP3CB*	0.0040	2.18
ENSG00000173786	*CNP*	0.0040	1.95
ENSG00000257151	*RP11-701H24.2.1*	0.0040	1.68
ENSG00000182195	*LDOC1*	0.0040	2.79
ENSG00000107105	*ELAVL2*	0.0040	3.80
ENSG00000168490	*PHYHIP*	0.0041	4.89
ENSG00000179915	*NRXN1*	0.0041	2.46
ENSG00000157152	*SYN2*	0.0044	5.61
ENSG00000162188	*GNG3*	0.0047	5.14
ENSG00000105649	*RAB3A*	0.0047	4.48
ENSG00000164061	*BSN*	0.0049	3.62
ENSG00000145920	*CPLX2*	0.0049	4.80
ENSG00000176381	*PRR18*	0.0050	6.45
ENSG00000167654	*ATCAY*	0.0050	3.54
ENSG00000070087	*PFN2*	0.0065	1.53
ENSG00000133169	*BEX1*	0.0070	3.77
ENSG00000177807	*KCNJ10*	0.0074	4.09
ENSG00000237289	*CKMT1B*	0.0086	5.46
ENSG00000136531	*SCN2A*	0.0086	2.57
ENSG00000008300	*CELSR3*	0.0086	1.98
ENSG00000047597	*XK*	0.0088	6.02
ENSG00000168314	*MOBP*	0.0088	2.73
ENSG00000171132	*PRKCE*	0.0091	2.36
ENSG00000130540	*SULT4A1*	0.0094	3.81
ENSG00000179292	*TMEM151A*	0.0095	5.12
ENSG00000101210	*EEF1A2*	0.0095	4.18
ENSG00000114646	*CSPG5*	0.0095	2.11
ENSG00000169851	*PCDH7*	0.0096	2.98
ENSG00000074317	*SNCB*	0.0103	5.91
ENSG00000166342	*NETO1*	0.0103	4.13
ENSG00000184611	*KCNH7*	0.0107	5.00
ENSG00000136928	*GABBR2*	0.0107	3.65
ENSG00000130287	*NCAN*	0.0107	3.47
ENSG00000078018	*MAP2*	0.0107	2.27
ENSG00000166165	*CKB*	0.0107	2.16
ENSG00000197959	*DNM3*	0.0108	1.52
ENSG00000254377	*RP11-32K4.2.1*	0.0115	4.49
ENSG00000171450	*CDK5R2*	0.0115	4.11
ENSG00000154146	*NRGN*	0.0120	4.22
ENSG00000018625	*ATP1A2*	0.0123	1.96
ENSG00000105409	*ATP1A3*	0.0123	4.50
ENSG00000104112	*SCG3*	0.0145	2.43
ENSG00000166257	*SCN3B*	0.0145	3.00
ENSG00000176884	*GRIN1*	0.0146	4.65
ENSG00000136854	*STXBP1*	0.0149	2.98
ENSG00000187189	*TSPYL4*	0.0149	1.79
ENSG00000173320	*STOX2*	0.0150	1.70
ENSG00000168280	*KIF5C*	0.0155	2.10
ENSG00000084628	*NKAIN1*	0.0156	3.61
ENSG00000126861	*OMG*	0.0174	4.17
ENSG00000157064	*NMNAT2*	0.0174	2.54
ENSG00000166448	*TMEM130*	0.0175	3.94
ENSG00000100146	*SOX10*	0.0175	2.76
ENSG00000186231	*KLHL32*	0.0175	4.10
ENSG00000145864	*GABRB2*	0.0179	4.31
ENSG00000177301	*KCNA2*	0.0179	3.95
ENSG00000103034	*NDRG4*	0.0179	1.93
ENSG00000118160	*SLC8A2*	0.0187	4.04
ENSG00000099822	*HCN2*	0.0187	3.43
ENSG00000109107	*ALDOC*	0.0187	3.22
ENSG00000165152	*C9orf125*	0.0197	3.66
ENSG00000144290	*SLC4A10*	0.0197	4.42
ENSG00000197177	*GPR123*	0.0197	4.18
ENSG00000172995	*ARPP21*	0.0197	2.71
ENSG00000101445	*PPP1R16B*	0.0201	2.61
ENSG00000198794	*SCAMP5*	0.0201	2.41
ENSG00000243156	*MICAL3*	0.0201	1.42
ENSG00000125648	*SLC25A23*	0.0201	1.31
ENSG00000105613	*MAST1*	0.0208	3.55
ENSG00000224189	*AC009336.23.1*	0.0221	5.47
ENSG00000188191	*PRKAR1B*	0.0221	2.60
ENSG00000084731	*KIF3C*	0.0221	1.92
ENSG00000075340	*ADD2*	0.0221	1.55
ENSG00000185046	*ANKS1B*	0.0224	1.87
ENSG00000165388	*ZNF488*	0.0229	4.90
ENSG00000155980	*KIF5A*	0.0229	3.29
ENSG00000113327	*GABRG2*	0.0230	5.57
ENSG00000130558	*OLFM1*	0.0232	4.28
ENSG00000109654	*TRIM2*	0.0232	1.52
ENSG00000159409	*CELF3*	0.0233	4.10
ENSG00000168243	*GNG4*	0.0233	3.52
ENSG00000008735	*MAPK8IP2*	0.0233	3.18
ENSG00000171617	*ENC1*	0.0233	2.36
ENSG00000196361	*ELAVL3*	0.0233	2.35
ENSG00000167123	*CERCAM*	0.0238	1.73
ENSG00000178233	*TMEM151B*	0.0240	3.08
ENSG00000173898	*SPTBN2*	0.0240	2.81
ENSG00000163032	*VSNL1*	0.0240	3.83
ENSG00000122966	*CIT*	0.0249	1.97
ENSG00000145087	*STXBP5L*	0.0252	2.99
ENSG00000171532	*NEUROD2*	0.0253	4.89
ENSG00000067715	*SYT1*	0.0263	3.98
ENSG00000197971	*MBP*	0.0263	3.14
ENSG00000054356	*PTPRN*	0.0263	2.34
ENSG00000134709	*HOOK1*	0.0265	5.09
ENSG00000127585	*FBXL16*	0.0268	2.81
ENSG00000073464	*CLCN4*	0.0268	2.01
ENSG00000165868	*HSPA12A*	0.0273	2.43
ENSG00000196338	*NLGN3*	0.0283	1.40
ENSG00000259969	*RP11-999E24.3.1*	0.0287	5.43
ENSG00000107295	*SH3GL2*	0.0287	4.27
ENSG00000019505	*SYT13*	0.0287	4.19
ENSG00000164742	*ADCY1*	0.0287	1.94
ENSG00000260918	*RP11-731J8.2.1*	0.0293	3.87
ENSG00000151150	*ANK3*	0.0293	2.24
ENSG00000008277	*ADAM22*	0.0293	1.37

ENSG00000123560	*PLP1*	0.0297	3.17
ENSG00000221823	*PPP3R1*	0.0297	1.42
ENSG00000020129	*NCDN*	0.0297	2.10
ENSG00000170579	*DLGAP1*	0.0302	3.10
ENSG00000112379	*KIAA1244*	0.0302	1.60
ENSG00000149927	*DOC2A*	0.0306	3.59
ENSG00000250686	*RP1-240B8.3.1*	0.0326	5.25
ENSG00000128872	*TMOD2*	0.0332	1.56
ENSG00000113763	*UNC5A*	0.0334	3.26
ENSG00000006116	*CACNG3*	0.0342	4.44
ENSG00000110786	*PTPN5*	0.0342	3.82
ENSG00000100095	*SEZ6L*	0.0342	3.22
ENSG00000154917	*RAB6B*	0.0342	2.06
ENSG00000187391	*MAGI2*	0.0342	1.43
ENSG00000172508	*CARNS1*	0.0346	2.48
ENSG00000175874	*CREG2*	0.0354	3.65
ENSG00000177108	*ZDHHC22*	0.0357	3.44
ENSG00000157087	*ATP2B2*	0.0357	3.20
ENSG00000255571	*CTD-2335A18.1.1*	0.0357	2.78
ENSG00000110881	*ACCN2*	0.0357	2.34
ENSG00000144230	*GPR17*	0.0370	4.44
ENSG00000126950	*TMEM35*	0.0370	4.38
ENSG00000123119	*NECAB1*	0.0380	3.37
ENSG00000156298	*TSPAN7*	0.0388	1.90
ENSG00000150672	*DLG2*	0.0391	2.07
ENSG00000106123	*EPHB6*	0.0403	3.73
ENSG00000087258	*GNAO1*	0.0403	3.07
ENSG00000050030	*KIAA2022*	0.0403	2.52
ENSG00000067606	*PRKCZ*	0.0411	2.52
ENSG00000122584	*NXPH1*	0.0420	4.35
ENSG00000078328	*RBFOX1*	0.0426	3.13
ENSG00000196090	*PTPRT*	0.0426	2.97
ENSG00000166501	*PRKCB*	0.0426	2.38
ENSG00000136960	*ENPP2*	0.0433	2.40
ENSG00000172137	*CALB2*	0.0433	5.88
ENSG00000099308	*MAST3*	0.0437	1.84
ENSG00000154027	*AK5*	0.0437	3.24
ENSG00000110076	*NRXN2*	0.0437	1.97
ENSG00000076826	*CAMSAP3*	0.0439	4.53
ENSG00000185760	*KCNQ5*	0.0442	2.87
ENSG00000224223	*GS1-18A18.1.1*	0.0455	5.94
ENSG00000180440	*SERTM1*	0.0460	4.64
ENSG00000130294	*KIF1A*	0.0461	3.08
ENSG00000110400	*PVRL1*	0.0466	2.18
ENSG00000128594	*LRRC4*	0.0466	2.17
ENSG00000139915	*MDGA2*	0.0466	4.29
ENSG00000114279	*FGF12*	0.0466	3.41
ENSG00000163539	*CLASP2*	0.0466	1.65
ENSG00000167971	*CASKIN1*	0.0485	3.12
ENSG00000123091	*RNF11*	0.0485	1.55
ENSG00000118276	*B4GALT6*	0.0486	4.08
ENSG00000122733	*KIAA1045*	0.0493	3.90
ENSG00000184144	*CNTN2*	0.0493	2.94
ENSG00000185742	*C11orf87*	0.0496	3.89
ENSG00000007237	*GAS7*	0.0496	1.29
ENSG00000018236	*CNTN1*	0.0496	3.66
ENSG00000186472	*PCLO*	0.0496	2.97

Table S4. Gene set enrichment analysis of up-regulated genes

GeneSet_ID (Gene Ontology)	*P*-Value	Odds ratio	False discovery rate
GO_nervous_system_development	7.76E-23	5.1	3.18E-19
GO_modulation_of_synaptic_transmission	1.55E-18	12.5	2.55E-15
GO_neuron_projection_development	3.71E-16	5.2	3.81E-13
GO_neurogenesis	2.05E-15	4.3	1.87E-12
GO_neuron_differentiation	4.67E-15	4.4	3.83E-12
GO_cognition	3.94E-14	10.6	2.49E-11
GO_neurotransmitter_transport	3.00E-13	12.1	1.54E-10
GO_cell_development	2.61E-12	3.5	1.13E-09
GO_synaptic_vesicle_localization	3.78E-12	14.0	1.55E-09
GO_neuron_projection_morphogenesis	1.13E-11	4.6	3.77E-09

GeneSet_ID (Gene lists)	*P*-Value	Odds ratio	False discovery rate
c2_MIKKELSEN_MEF_HCP_WITH_H3K27ME3	9.38E-33	16.7	7.70E-29
c2_BLALOCK_ALZHEIMERS_DISEASE_DN	8.54E-21	5.6	1.75E-17
c2_VERHAAK_GLIOBLASTOMA_PRONEURAL	7.10E-21	17.2	1.75E-17
c2_YOSHIMURA_MAPK8_TARGETS_UP	4.04E-18	5.8	5.54E-15
c3_V$NRSF_01	6.06E-17	28.1	7.11E-14
c6_KRAS.KIDNEY_UP.V1_UP	8.70E-15	15.2	6.50E-12
c2_MIKKELSEN_MCV6_HCP_WITH_H3K27ME3	3.45E-14	9.3	2.36E-11
c2_REACTOME_NEURONAL_SYSTEM	7.66E-14	10.2	4.49E-11
c2_KIM_ALL_DISORDERS_CALB1_CORR_UP	1.61E-13	6.0	8.81E-11
c2_BENPORATH_ES_WITH_H3K27ME3	8.83E-13	5.1	4.27E-10

Table S5. Gene set enrichment analysis of down-regulated genes

GeneSet_ID (Gene Ontology)	*P*-Value	Odds ratio	False discovery rate
GO_blood_vessel_development	2.02E-19	17.4	2.37E-16
GO_vasculature_development	5.70E-19	16.5	5.85E-16
GO_cardiovascular_system_development	9.18E-16	11.1	6.28E-13
GO_circulatory_system_development	9.18E-16	11.1	6.28E-13
GO_collagen_metabolic_process	2.79E-13	35.3	1.04E-10
GO_endodermal_cell_differentiation	2.89E-12	71.7	9.14E-10
GO_endoderm_formation	9.29E-12	60.5	2.54E-09
GO_skeletal_system_development	1.52E-11	11.8	4.02E-09
GO_collagen_fibril_organization	2.45E-10	57.5	4.47E-08
GO_cartilage_development	6.97E-10	19.4	1.15E-07

GeneSet_ID (Gene list)	*P*-Value	Odds ratio	False discovery rate
ch_HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	1.22E-25	41.5	1.00E-21
c2_VERHAAK_GLIOBLASTOMA_MESENCHYMAL	2.67E-24	35.5	1.10E-20
c2_SCHUETZ_BREAST_CANCER_DUCTAL_INVASIVE_UP	5.08E-23	25.5	1.39E-19
c2_PID_INTEGRIN1_PATHWAY	1.48E-22	86.5	3.05E-19
c2_NABA_MATRISOME	4.76E-22	17.6	7.82E-19
c2_KEGG_ECM_RECEPTOR_INTERACTION	4.32E-21	66.7	5.91E-18
c2_NABA_CORE_MATRISOME	6.05E-17	24.5	5.52E-14
c2_PICCALUGA_ANGIOIMMUNOBLASTIC_LYMPHOMA_UP	3.23E-16	25.0	2.65E-13
c2_ANASTASSIOU_CANCER_MESENCHYMAL_TRANSITION_SIGNATURE	2.56E-15	56.9	1.62E-12
c2_CHARAFE_BREAST_CANCER_LUMINAL_VS_MESENCHYMAL_DN	4.73E-15	13.9	2.78E-12

Table S6. Ingenuity Pathway Analysis of activated and inhibited canonical pathways

TOP CANONICAL PATHWAYS	*P*-value	Ratio	Z-score	Molecules
G Beta Gamma Signaling	$1.13 \cdot 10^{-4}$	9.9 % 8/81	2.1	ADCY1,PRKCE,GNAO1,GNG3,GNG4,PRKCB, PRKAR1B,PRKCZ
Role of NFAT in Cardiac Hypertrophy	$6.52 \cdot 10^{-4}$	6.3 % 10/158	2.8	ADCY1,PRKCE,SLC8A2,PPP3R1,GNG3,GNG4,PPP3CB, PRKCB,PRKAR1B,PRKCZ
Synaptic Long Term Potentiation	$6.75 \cdot 10^{-4}$	7.6 % 8/105	2.8	ADCY1,PRKCE,PPP3R1,PPP3CB,PRKCB,PRKAR1B, PRKCZ,GRIN1
Dopamine-DARPP32 Feedback in cAMP Signaling	$9.26 \cdot 10^{-4}$	6.6 % 9/137	2.1	ADCY1,PRKCE,PPP3R1,PPP3CB,PRKCB,PRKAR1B, KCNJ10,PRKCZ,GRIN1
Androgen Signaling	$2.11 \cdot 10^{-3}$	7.1 % 7/98	2.0	GNAO1,GNG3,GNG4,PRKAR1B,PRKCB,PRKCE,PRKCZ
CREB Signaling in Neurons	$2.19 \cdot 10^{-3}$	5.8 % 9/155	2.4	ADCY1,PRKCE,GNAO1,GNG3,GNG4,PRKCB, PRKAR1B,PRKCZ,GRIN1
P2Y Purigenic Receptor Signaling Pathway	$3.65 \cdot 10^{-3}$	6.5 % 7/108	2.2	ADCY1,PRKCE,GNG3,GNG4,PRKCB,PRKAR1B,PRKCZ
Melatonin Signaling	$4.08 \cdot 10^{-3}$	8.6 % 5/58	2.2	PRKCE,GNAO1,PRKCB,PRKAR1B,PRKCZ
Corticotropin Releasing Hormone Signaling	$6.42 \cdot 10^{-3}$	6.6 % 6/91	2.4	ADCY1,PRKCE,GNAO1,PRKCB,PRKAR1B,PRKCZ
IL-3 Signaling	$7.08 \cdot 10^{-3}$	7.6 % 5/66	2.2	PRKCE,PPP3R1,PPP3CB,PRKCB,PRKCZ
Calcium Signaling	$7.40 \cdot 10^{-3}$	5.7 % 7/123	2.2	ATP2B2,SLC8A2,PPP3R1,PPP3CB,PRKAR1B,ACTA2, GRIN1
GNRH Signaling	$2.20 \cdot 10^{-2}$	5.0 % 6/119	2.4	ADCY1,PRKCE,PRKCB,PRKAR1B,PRKCZ,DNM3
Neuropathic Pain Signaling In Dorsal Horn Neurons	$2.25 \cdot 10^{-2}$	5.7 % 5/88	2.2	PRKCE,PRKCB,PRKAR1B,PRKCZ,GRIN1
Renin-Angiotensin Signaling	$3.51 \cdot 10^{-2}$	5.1 % 5/99	2.2	ADCY1,PRKCE,PRKCB,PRKAR1B,PRKCZ
Integrin Signaling	$4.64 \cdot 10^{-2}$	3.9 % 7/179	-2.2	ITGA1,ACTA2,TSPAN7,ARPC1B,PFN2,ITGA5,ITGA7
eNOS Signaling	$4.96 \cdot 10^{-2}$	4.6 % 5/109	2.2	ADCY1,PRKCE,PRKCB,PRKAR1B,PRKCZ

Table S7. Ingenuity Pathway Analysis of activated and inhibited up-stream regulators

UPSTREAM REGULATORS	*P*-value	Z-score	Down-stream effector molecules
TGFB1	8.36E-10	-4.0	TIMP1, TGFBI, SERPINH1, SERPINE1, MMP13, ITGA5, ITGA1, IGFBP3, HOOK1, HMOX1, FN1, ENG, COL6A3, COL5A1, COL3A1, COL1A2, COL1A1, CDKN1A, BGN, ACTA2, TSPAN7, THBS1
ERBB2	6.75E-03	-2.4	ITGA5, FN1, COL6A3, COL5A, COL1A1, CDKN1A, THBS1
ERK	6.13E-03	-2.2	THBS1, SERPINE1, MMP14, MMP13, COL1A1
SMAD3	3.88E-06	-2.2	MMP13, FN1, COL3A1, COL1A1, COL1A2, COL1A1, CDKN1A, SERPINE1
estrogen receptor	1.27E-04	2,1	CALB2, COL4A1, COL4A2, COL5A1, FGF12, FN1, LOXL2, MMP14, PCDH7, SERPINE1, TIMP1
SPDEF	2.92E-05	2,6	COL1A1, COL4A1, COL4A2, COL5A1, COL6A3, ITGA5, SERPINE1

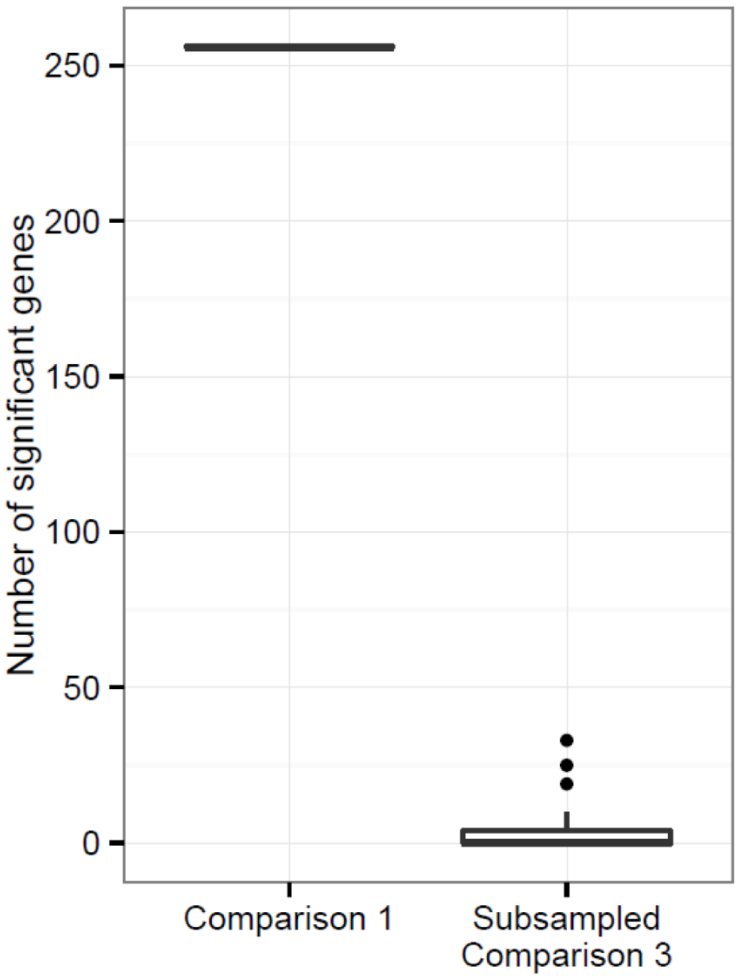

Figure S1. Subsampling analysis. This analysis subsampled pairs of non-responders (Comparison 3) to random groups of 6 patients 100 times. Comparison 1 and Comparison 3 show the number of differentially expressed genes in the paired comparison analysis of responders and non-responders, respectively.

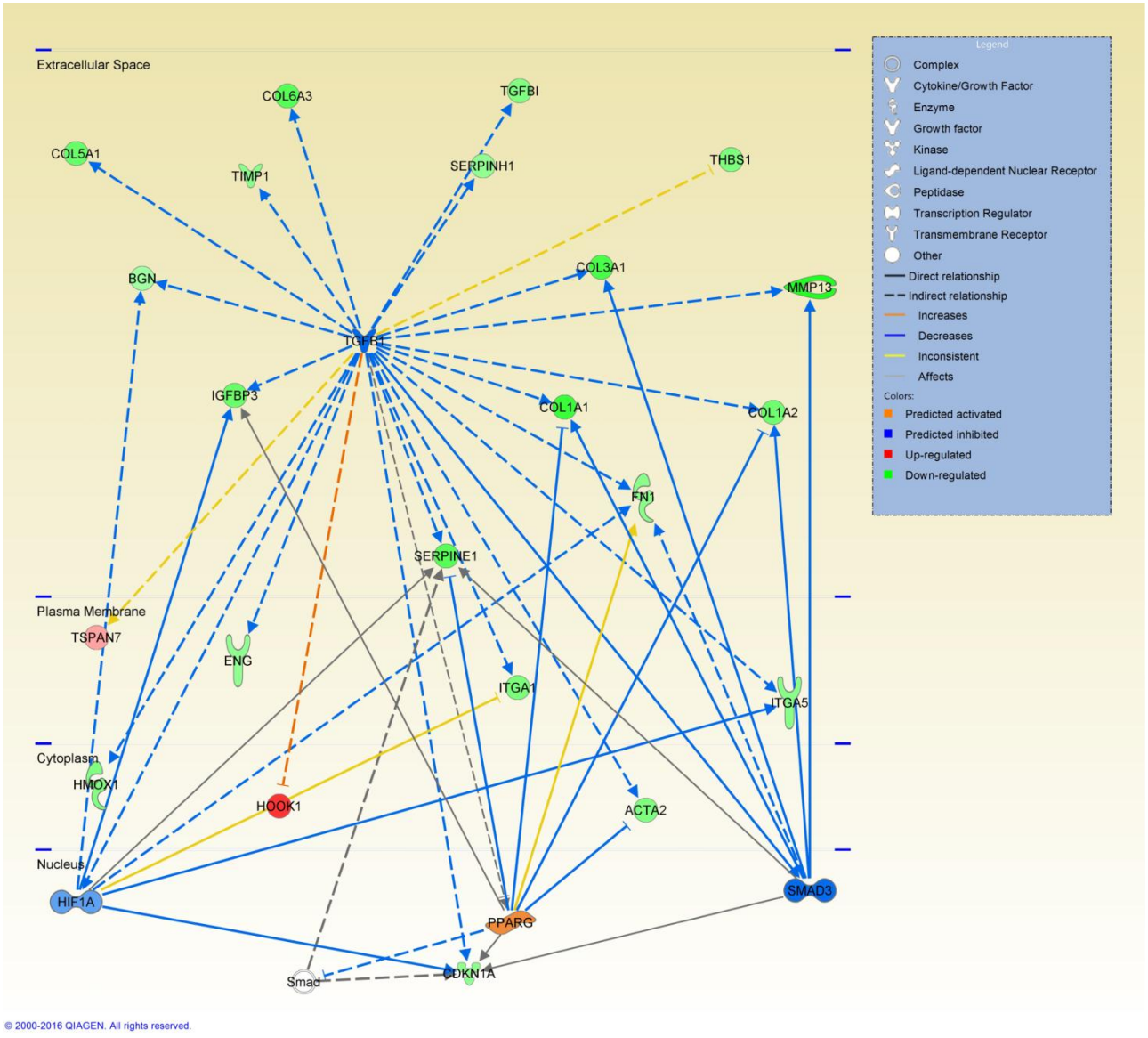

Figure S2. Mechanistic network of inhibited TGF-β1 and the three most interconnected regulators (SMAD3, HIF1A and PPARG) of downstream molecules identified differentially expressed in responders at the time of progression.

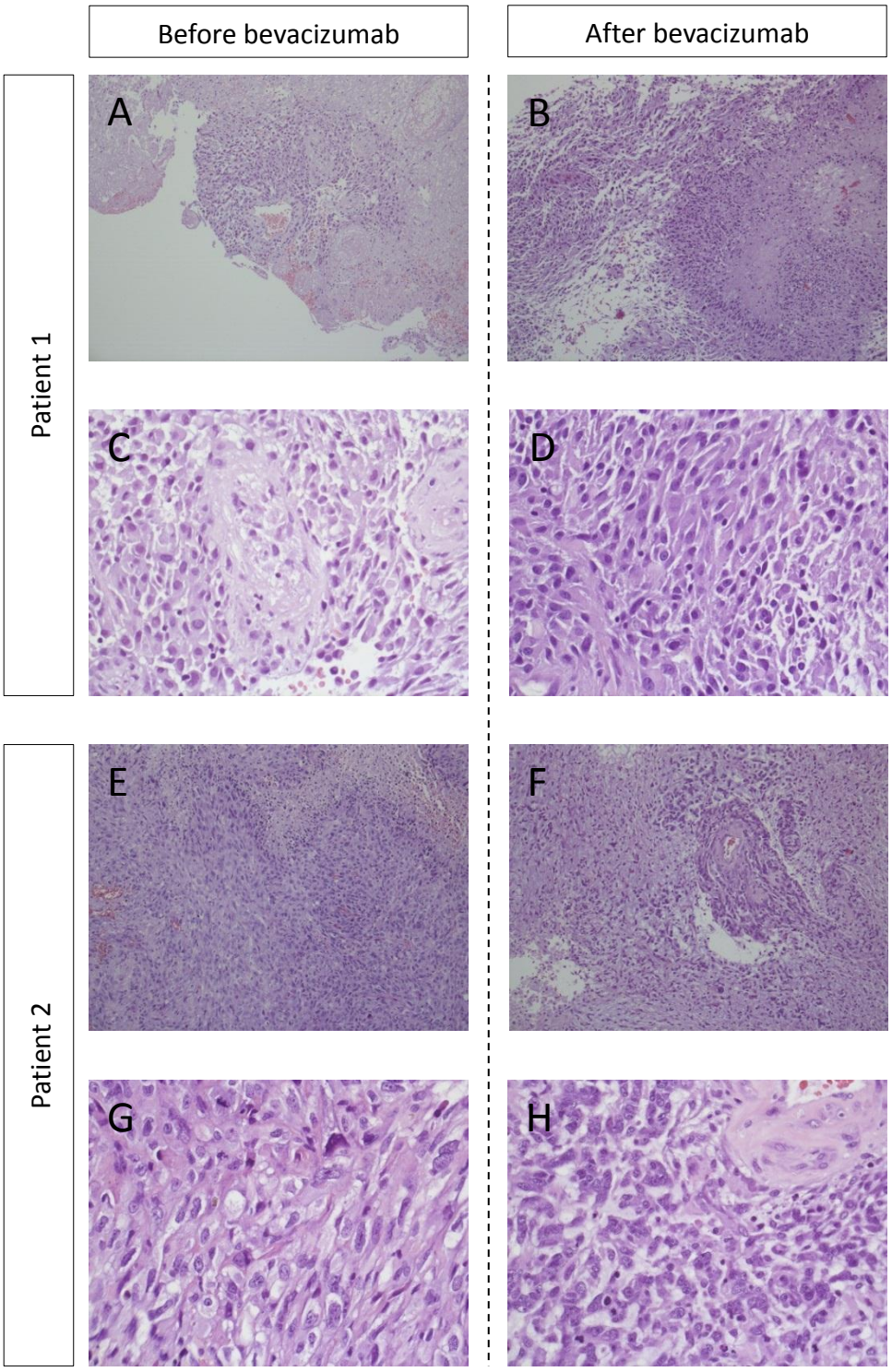

Figure S3. Hematoxylin-eosin staining of two representative responding glioblastomas before and after bevacizumab therapy. Overviews (×100) are shown in A, B, E, F and perivascular areas (×400) of corresponding samples are shown below in C, D, G, H.

4. DISCUSSION

4.1 Considerations concerning methodology

4.1.1 Study populations

This thesis is based on three retrospective studies of recurrent glioblastoma patients treated with bevacizumab and irinotecan. The studies include overlapping cohorts of patients treated with this combination therapy according to the same treatment protocol.[64]

In **Study I**, all patients treated with bevacizumab and irinotecan within the study period were included in the training dataset (initial cohort), including 21 patients treated in a phase-II trial.[51] The validation cohort consisted of patients treated at another center. The cohorts were comparable in terms of prognosis and we believe that they represent the general population of recurrent glioblastoma patients treated outside clinical trials.

In **Study II-III**, patients who were administered bevacizumab and irinotecan in combination with cetuximab in a phase 2 trial were also assessed for eligibility.[52] Several patients were excluded, primarily due lack of accessible tumor tissue. This may have introduced a selection bias.

4.1.2 Response

Treatment response is normally evaluated by the Macdonald criteria or the more recently established Revised Assessment in Neuro-Oncology (RANO) criteria.[65;66] In short, both response assessment criteria comprise changes in clinical status, corticosteroid dose and contrast-enhancing tumor measurements. The more recently established RANO criteria also include changes in non-enhancing T2-weighted/fluid-attenuated inversion recovery sequences (FLAIR).

At our center response assessment has been implemented as part of the clinical routine and our clinical database is frequently being updated by means of neuro-radiological reports (contrast-enhancing tumor measurements) and clinical records (corticosteroid dose and clinical status). Clinical status is evaluated on the basis of WHO performance status and neurological symptoms scored in six different categories using a three-tier system (none = 0; minor = 1; moderate/severe = 2).

Response was evaluated by the Macdonald Criteria in **Study I** and the RANO criteria in **Study II**-**III**.

Response evaluation has several inherent difficulties.[49;66;67] One obstacle is that changes in MRI enhancement and non-enhancement are not always related to tumor progression or response. Especially anti-angiogenic agents can show signs of MRI response due to reduced leakiness of tumor vasculature and not necessarily antitumor activity. This represents a so-called pseudo-response. Consequently, it is recommended to confirm a possible response by MRI 4 weeks later.[66]

Evaluation of response as an efficacy endpoint:

In **Study I** we analyzed if response at first response evaluation was associated with PFS and OS. In order to adjust for immortal time bias up to the first response evaluation,[68] we performed a so-called landmark analysis from the time of first response evaluation (landmark: week 9). As shown in *Appendix I*, this analysis demonstrated that response at first evaluation was an independent predictor of PFS and OS. This is in line with previous findings, including a previous study performed on a subset of the same patients.[51;57;69]

In **Study I**, we investigated changes in clinical status according to best response categorized as response, stable disease or progressive disease (defined according to Macdonald). Changes from baseline to time of best response in corticosteroid dose (any change), WHO performance status, neurological status (sum of all neurological categories scored 0, 1 or 2) and neurocognitive deficit were compared to response, as shown in *Appendix II*. This analysis showed that 43% of patients with response or stable disease were reduced in corticosteroid dose. The majority of patients had unchanged performance status and neurological symptoms; however, for patients that did experience changes, responders had significantly improved performance status and neurological status compared to patients with stable disease.

4.1.3 Biomarker analysis: procedure and pitfalls

To identify biomarkers associated with treatment response we analyzed tumor tissue using gene expression analysis. Due to the lack of systematic collected fresh-frozen tissue, which is the gold

standard for gene expression analysis, we used archived formalin-fixed, paraffin-embedded (FFPE) tissue.

Tissue handling:

Protein coding RNA molecules in FFPE samples is subject to degradation, fragmentation and cross-linking, which make gene expression profiling more challenging relative to fresh-frozen tissue. Furthermore, RNA molecules are unstable and each step from surgical removal of the tumor to generation of expression data can affect the analytical output. Therefore, in **Study II-III** tissue handling and RNA extraction were performed by RNA experienced technicians. In **Study II** laser capture microdissection was performed in order to increase tumor cell frequency and reduce inter-tumor heterogeneity influenced by non-malignant cells. In **Study III** macro-dissection was performed in some cases to insure a relatively high tumor cell frequency and to allow inclusion of the microenvironment. RNA-extraction was performed manually in both studies. In **Study III** RNA-extraction was performed in paired batches of pre- and post-treatment samples to reduce possible batch-effect in the paired comparison analysis. In both studies, the extracted RNA was highly degraded and no limitation to the RNA quality was set.

Gene expression methods:

Several methods have been developed in recent years to analyze gene expression from FFPE tissue. Here we used two of the leading technologies called NanoString and RNA-sequencing which have shown high performance on archived FFPE tumor tissue.[58;59;70] Both methods use small amounts of total RNA as input and follow relatively simple and standardized protocols. NanoString can quantify up to 800 transcripts by counting customized barcode probe/RNA complexes. RNA-sequencing is a Next-Generation Sequencing technology which is able to sequence and quantify present transcripts without preselecting a panel of genes.

The NanoString dataset showed no signs of batch-effect and was easily normalized by the use of reference genes. However, the RNA-sequencing dataset showed batch-effect in the comparison of pretreatment samples (comparison 2). This was due to different RNA-extraction dates and library

preparation dates (gender, primary/relapse surgery and age of archived tissue showed no batch-effect).

4.1.4 Statistical and bioinformatic considerations

All three studies in this thesis were based on limited knowledge regarding factors associated with bevacizumab response. Consequently, we decided to screen multiple factors for association with response. This was performed by using highly conservative statistical designs and methods in order to reduce the number of false positive findings and to objectively identify factors of interest.

4.2 Overall discussion

The findings of this thesis have generated new knowledge in relation to patient and tumor heterogeneity and how these factors impact response to bevacizumab combination therapy in recurrent glioblastoma patients. In addition, new hypotheses have been established regarding the mechanisms of response and acquired resistance to bevacizumab therapy.

In **Study I**, we identified three independent poor prognostic factors, namely corticosteroid use, multifocal disease and neurocognitive deficit. Based on these factors a prognostic model was established and validated in an independent dataset. This model shows that some patients have an extremely poor prognosis and are unlikely to benefit from therapy. Consequently, this prognostic model has been implemented in the clinic at our center for treatment planning.

Due to the difficulties in evaluating response and progression-free survival in anti-angiogenic treated glioblastoma patients, OS is by many clinical investigators considered the best primary endpoint in recurrent glioblastoma patients.[71] However, in **Study I** we demonstrated that OS is highly variable, even within known prognostic groups. This indeed complicates the interpretation of OS as an efficacy endpoint.

In contrast to PFS and OS, we showed in **Study I** that clinical factors had limited impact on response. Corticosteroid use was associated with response in the initial cohort, but this was not confirmed in the validation cohort. In addition, response was significantly associated with longer PFS and OS and to some extent improved clinical status. Therefore, we believe that response is a preferable endpoint for

measuring the activity of anti-angiogenic agents in the highly heterogeneous population of recurrent glioblastoma patients.

In **Study II,** we identified two predictive biomarkers for response, namely low gene expression of angiotensinogen and high expression of a HLA-class II gene (*HLA-DQA1*). Both were included in a predictive model for bevacizumab response (Figure 5).

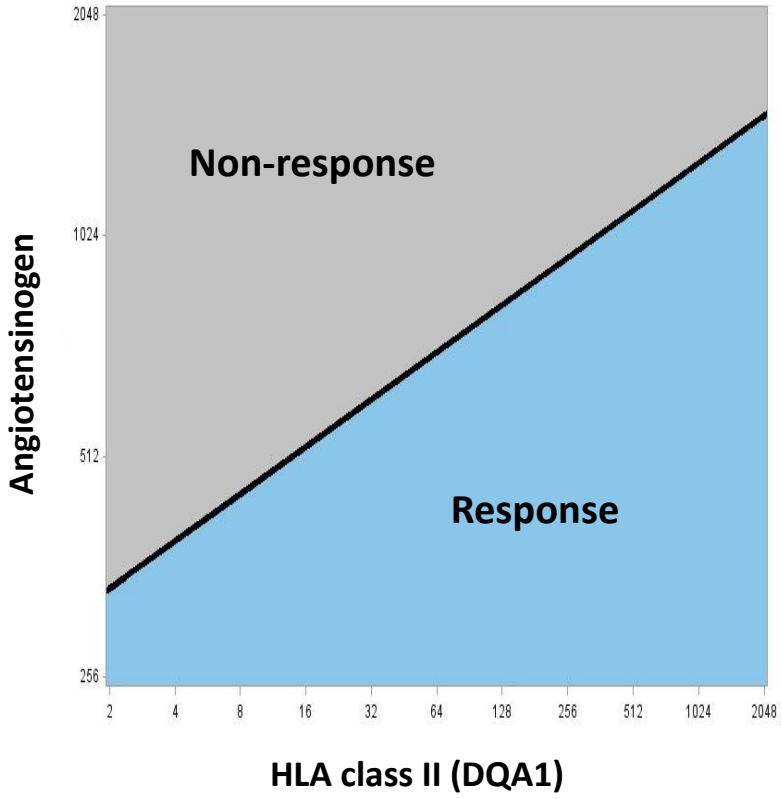

Figure 5. The predictive model for response established in **Study II**.

This model offers simple interpretation and can be used in clinical practice. As an example, if a patient has a tumor with a high expression of angiotensinogen and low expression of the HLA-class II gene, the patient will most likely not respond to bevacizumab combination therapy. This model can be used to distinguish between patients who will or will not benefit from bevacizumab combination therapy. However, in order to be implemented in the clinical setting it will have to be validated.[72]

NanoString is a relatively reliable method (EU and FDA approved in breast cancer, Prosigna). However, it would be desirable to establish a simpler method closer to the current clinical routine. Therefore we

analyzed the identified biomarkers by immunohistochemistry. Here, we confirmed the protein expression of both angiotensinogen and HLA-class II in glioblastoma. However, we were unable to establish a quantifiable method for angiotensinogen based on the analysis. This was in part due to the inter-tumor heterogeneity but especially the diffuse localization of angiotensinogen. However, we did observe a marked increase in vascular fibrosis and remodeling in tumors with high angiotensinogen gene expression. We speculated that this could be related to angiotensin-II induced remodeling and fibrosis, which recently has been suggested as a resistance mechanism to anti-angiogenic therapy in preclinical studies.[73] Based on our findings and the literature, we hypothesized that:

- Angiotensinogen promotes resistance to bevacizumab-induced vascular normalization through angiotensin-II-mediated tissue remodeling effects on the vasculature.
- HLA-class II reflects an anti-tumor immune system which is maintained or activated by bevacizumab-induced vascular normalization.

In **Study III**, the objective was to identify differentially expressed genes in a paired comparison analysis of responding and non-responding glioblastomas. The results of the analysis showed significantly transcriptional changes in responders, but only minimal changes in non-responding glioblastomas. In our opinion, this suggests that responding glioblastomas adaptively change as they respond or progress, while non-responding glioblastomas progress unaffected by the treatment.

In **Study III,** we performed bioinformatic analyses based on published data in order to identify possible response and acquired resistance mechanisms related to the gene changes observed in bevacizumab-responding patients. Most of the identified mechanisms were, in our view, mostly related to response mechanisms rather than resistance mechanisms (Figure 6). However, protein kinase C signaling was found transcriptionally activated. This pathway has been described as an alternative angiogenic signaling pathway,[74] and may be involved in acquired resistance to bevacizumab therapy. Nevertheless, protein kinase C inhibition has not proven active in unselected glioblastoma patients,[75-77] though bevacizumab in combination with an agent inhibiting this pathway may be effective in glioblastoma patients responding to bevacizumab.

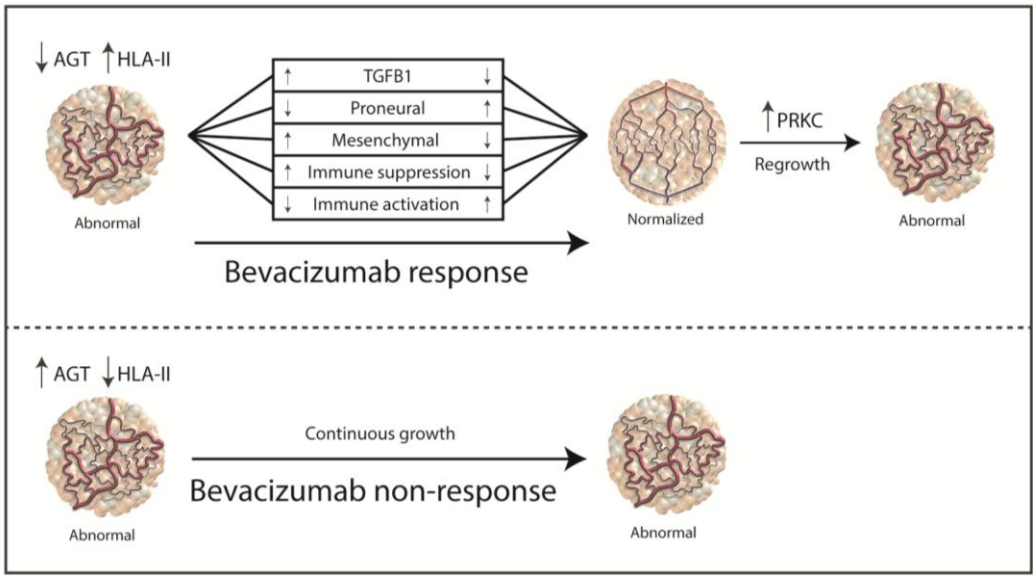

Figure 6. Working hypothesis for bevacizumab response and resistance based on results from **Study II**-**III**. Modified from Huang et al.[36]

In **Study III**, we observed that responding tumors progress phenotypically as less mesenchymal and more proneural, according to the molecular classification systems.[19;20] These results are in contrast to most preclinical animal studies using transplanted patient-derived tumor models (xenograft).[78;79] The contrasting differences may be explained by the evident differences between the clinical setting and preclinical studies, including dosing and scheduling of anti-angiogenic agents.[28;80-82] Another possibility is that only a fraction of the tested xenograft models originate from patient tumors sensitive to bevacizumab therapy.

In **Study III,** we found indications of inactivated TGF-β signaling in responders, possibly related to bevacizumab induced vascular normalization. TGF-β has a central role in induction of immunosuppression in glioblastoma.[83] Thus, inhibiting TGF-β in responding patients may tip the balance in favor of a more activate anti-tumor immune system. This may explain the association of HLA-class II positive antigen-presenting cells and bevacizumab response observed in **Study II**. In line with previous studies,[36] this supports the rationale of combining bevacizumab with immunomodulating agents in ongoing and future clinical trials.

5. CONCLUSIONS

This thesis has studied clinical factors and biomarkers for association with bevacizumab response and resistance in recurrent glioblastoma patients. This has generated the following conclusions:

- Three prognostic factors of early progression and mortality were identified: Corticosteroid use, multifocal disease and neurocognitive deficit. Based on these factors a prognostic model was developed and validated in an independent dataset. This model can be used in the clinic for treatment planning.
- Response reflects bevacizumab activity and should be considered an early efficacy endpoint for anti-angiogenic agents in recurrent glioblastoma patients.
- Two predictive biomarkers for response were identified: Low gene expression of angiotensinogen and high expression of a HLA-class II gene. These two factors were incorporated in a predictive model for response. If validated, this model can identify patients who will or will not benefit from bevacizumab combination therapy.
- Glioblastomas not responding to bevacizumab therapy progress by means of intrinsic resistance, perhaps related to angiotensin-II mediated effects on the tumor microenvironment.
- Glioblastomas responding to bevacizumab therapy progress by means of acquired resistance. Activated protein kinase C signaling may be involved in these mechanisms.
- Glioblastomas responding to bevacizumab therapy respond by adaptive mechanisms. These mechanisms are related to TGF-β inhibition which may activate an anti-tumor immune system.

6. PERSPECTIVES

The first priority for future studies is to validate the predictive model. If we can validate, that a defined subgroup of recurrent glioblastoma patients benefit from bevacizumab combination treatment, this will change the currently unselected treatment planning and improve the personalization of therapy for this patient population.

It would be preferable to perform a validation study on an independent cohort of patients from one of the published randomized trials.[58-60] However, several differences between patient cohorts, treatment regimens, biomarker assessment methods and possible batch-effects have to be taken into consideration before performing such an analysis.

In order to validate our findings we performed immunohistochemical analysis but this method was not suitable for quantification. Utilizing the same methods as in **Study II** will be the most appropriate for a validation study. However, we are also considering other approaches in order to perform a more simple quantification of the biomarkers.

An alternative validation study is a prospective trial using a personalized medicine treatment protocol which will be initiated at our center. This study design will demand pre-specified biomarker-based eligibility criteria and will allow testing of new agents in combination with bevacizumab therapy. Such combinatory treatments could potentially be with immunomodulating agents or protein kinase C inhibitors.

For future studies it will be of importance to establish an objective and robust method to measure early efficacy of agents tested in glioblastoma patients. Plasma biomarkers and imaging techniques are currently being studied in this regard. Recent results suggest that positron emission tomography (PET) efficiently can measure early response to bevacizumab therapy.[84] This may also open up for other more specific PET-markers in the response evaluation.[85]

One of the main reasons for the lack of validated predictive biomarkers is our limited understanding of response and resistance mechanisms to bevacizumab therapy. Therefore, an improved characterization of these mechanisms will be valuable for future clinical studies. In this regard we have planned the following: 1) Validation studies of the results from the paired sample analyses in

Study III. 2) Systematic tissue banking of high quality patient samples for future studies. 3) Optimization of preclinical models and preclinical study designs in order to resemble the clinical setting.

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8. APPENDICES

8.1 Appendix I: Multivariate analysis of response as a predictor for PFS and OS from first response evaluation (landmark week 9).

Variable	**PFS from week 9 HR (95% CI)**	**OS from week 9 HR (95% CI)**
WHO PS at landmark, 1 vs. 0	1.37 (0.94-2.00) *P*=0.11	1.37 (0.93-2.01) *P*=0.11
WHO PS at landmark, ≥2 vs. 0	1.76 (1.06-2.91) *P*=0.03	1.91 (1.19-3.07) *P*<0.01
Neurocogntive deficit at baseline, Yes vs. no	1.11 (0.81-1.52) *P*=0.53	1.23 (0.89-1.69) *P*=0.21
Multifocal at baseline, yes vs. no	1.85 (1.31-2.62) *P*<0.001	2.00 (1.42-2.81) *P*<0.001
Use of corticosteroids[a] at landmark, yes vs. no	1.03 (0.71-1.48) *P*=0.87	1.45 (1.00-2.10) *P*=0.05
Response at landmark, SD+PD vs. CR+PR	1.45 (1.01-2.07) *P*=0.04	1.55 (1.06-2.26) *P*=0.02
C-index	0.68	0.70

Abbreviations: PFS, progression-free survival; OS, overall survival; HR, hazards ratio; WHO PS, WHO performance status; SD, stable disease; PD, progressive disease; CR, complete response; PR, partial response; C-index, concordance index.
Note: Only response evaluable patients from Population 1 were included.
[a] Prednisolone >10mg.

8.2 Appendix II: Changes in clinical status according to best response.

Changes in clinical status at best response	CR+PR (*n*=66)	SD (*n*=108)	PD (*n*=23)
Corticosteroid dose[a]; *n* (%)			
Decreased	28 (43)	45 (43)	4 (19)
Unchanged	34 (52)	46 (43)	7 (33)
Increased	3 (5)	15 (14)	10 (48)
NA	1	2	2
WHO PS[a]; *n* (%)			
Improved	10 (91)	10 (37)	1 (9)
Worsened	1 (9)	17 (63)	10 (91)
NA	0	2	1
Neurological status[a]; *n* (%)			
Improved	12 (86)	12 (41)	0 (0)
Worsened	2 (14)	17 (59)	12 (100)
NA	3	5	0
Neurocogntive status[a]; *n* (%)			
Improved	5 (71)	5 (42)	0 (0)
Worsened	2 (29)	7 (58)	8 (100)
NA	3	5	0

Abbreviations: WHO PS, WHO performance status; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. NA, not available.

Note: Only response evaluable patients from Population 1 were included. Patients with unchanged WHO PS and neurological symptoms were excluded from percent determination and statistics. Missing data were not included in percent determination and statistics.

[a]Changes were defined as any change in corticosteroid dose, WHO PS and neurological status (sum of all 6 neurological categories. Categories are listed in Suppl. Table S1 in Study I).

“It is very difficult to predict — especially the future.”

Niels Bohr