Human complement factor H is a novel diagnostic marker for lung adenocarcinoma

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ABBREVIATIONS

ADC	Adenocarcinoma
cDNA	Complementary deoxyribonucleic acid
CFH	Complement factor H
CFHL	CFH-like protein
CFHR	CFH-related protein
DNA	Deoxyribonucleic acid
FISH	Fluorescence in situ hybridization
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IHC	Immunohistochemistry
LCLC	Large cell lung cancer
MAC	Membrane attack complex
mRNA	Message ribonucleic acid
NSCLC	Non-small cell lung cancer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
RT-PCR	Reverse transcriptase polymerase chain reaction
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
TMA	Tissue microarray
TTF-1	Thyroid transcription factor-1

CONTENTS

SUMMARY

Background

Human complement factor H (CFH), a central complement control protein, is a member of the regulators of complement activation family. Recent studies suggested that CFH may play a key role in resistance of complement mediated lysis in various cancer cells. In this study, we investigated the role of CFH in human lung cancer.

Methods

Expression of CFH in lung cancer cells was analyzed by RT-PCR, western blotting, and immunofluorescence. Binding of CFH to lung cancer cells was detected by flow cytometry. In primary lung tumors, the protein expression of CFH was evaluated by IHC on tissue microarray.

Results

We found, mRNA expression of CFH was detected in six out of ten NSCLC cell lines, but not in SCLC cell lines. Consistence with the western blotting result, immunofluorescence analysis demonstrated CFH protein expression in three NSCLC cell lines, and the immunoreaction was mainly associated with cell cytoplasm and membrane. In primary lung tumors, 54 out of 101 samples exhibited high expression of CFH, and high expression was significantly correlated with lung adenocarcinoma ($p=0.009$). Also, in ADC of lung, Kaplan-Meier survival analysis showed a tendency that CFH-positive tumors had worse prognosis in comparison to CFH-negative tumors ($p=0.082$). Additionally, shorter survival time of patients with ADC (less than 20 months) was associated with higher protein expression of CFH ($p=0.033$).

Conclusion

Our data showed that non-small cell lung cancer cells expressed and secreted CFH. CFH might be a novel diagnostic marker for human lung adenocarcinoma.

Zusammenfassung

Hintergrund

Der humane Komplement Faktor H (CFH), ein zentraler Regulator der Komplementkaskade gehört zur Familie der Komplement-Aktivatoren. Neuere Studien führen zu der Annahme, dass CFH eine wichtige Rolle bei der Verhinderung der Komplement-vermittelte Lyse in verschiedenen Krebszellen spielen kann. In dieser Studie untersuchten wir die Rolle von CFH im menschlichen Lungenkrebs.

Methoden

Die Expression von CFH durch Lungenkrebszellen wurde mittels RT-PCR, Western Blot und Immunfluoreszenz analysiert. Die Bindung von CFH an Lungenkrebs-Zellen wurde mittels Durchflusszytometrie nachgewiesen. An Hand primärer Lungentumore wurde die Proteinexpression von CFH immunhistochemisch an Gewebe-Mikroarrays untersucht.

Ergebnisse

Die Expression von CFH-mRNA war in sechs von zehn NSCLC-Zelllinien nachweisbar, nicht jedoch in SCLC-Zellinien. Im Einklang mit den Resultaten des Western-Blots belegte die Immunfluoreszenzanalytik CFH-Proteinexpression in drei NSCLC-Zelllinien, insbesondere zytoplasmatisch und membranassoziiert. Im primären Lungentumorgewebe zeigte sich in 54 von 101 Fällen eine hohe CFH-Expression, hohe Expressionsraten korrelierten signifikant mit dem Auftreten des Adenokarzinomsubtyps ($p = 0.009$). In der Kaplan-Meier-Analyse zeigte sich für das ADC ebenfalls die Tendenz einer Korrelation von CFH-Positivität und schlechter klinischer Prognose ($p = 0.082$). Des Weiteren ist eine kürzere Überlebenszeit von Patienten mit ADC (weniger als 20 Monate) mit stärkerer CFH-Färbung ($p = 0.033$) assoziiert.

Schlussfolgerungen

Unsere Daten belegen, dass nicht-kleinzellige Lungenkarzinomzellen CFH exprimieren und sezernieren. CFH könnte ein neuer diagnostischer Marker für das menschliche Adenokarzinom der Lunge werden.

INTRODUCTION

1. Classification of lung cancer

Lung cancer is the leading cause of cancer-related death worldwide (Jemal et al, 2008), and only 15% of all lung cancer patients are alive 5 years or more after diagnosis. Common symptoms of lung cancer include cough, dyspnoea, weight loss, and chest pain. Symptomatic patients are more likely to have obstructive pulmonary disease.

Lung cancers are classified into two main categories: small-cell lung cancer (SCLC), which accounts for approximately 20% of cases, and non-small cell lung cancer (NSCLC), which accounts for the other 80%. NSCLC includes squamous cell carcinoma (≈25%), adenocarcinoma (≈40%) and large cell carcinoma (≈15%). Squamous cell carcinomas (SCCs) arise preferentially from bronchi near the hilus with potential involvement of trachea and derive from stem cells of a dysplastic multilayer epithelium that underwent squamous metaplasia. Most cases of SCCs are associated with smoking. Well-differentiated squamous cell lung carcinomas often grow more slowly than other cancer types (Komaki et al, 2000). Adenocarcinomas (ADCs) tend to be located in the periphery of the lung and originate preferentially from precursor cells of the mono- or bilayer surface epithelium of the lung periphery. Among people who have never smoked ("never-smokers"), ADC is the most common form of lung cancer (Subramanian et al, 2007). A subtype of ADC, the bronchioloalveolar carcinoma, is more common in female never-smokers, and may have different responses to treatment (Raz et al, 2006). Large cell lung carcinoma (LCLC) is a heterogeneous group of undifferentiated malignant neoplasm originating from transformed epithelial cells in the lung. The newest revisions of the World Health Organization histological typing of lung cancer schema includes several variants of large cell carcinoma, such as (a) basaloid, (b) clear cell, (c) lymphoepithelioma-like, (d) rhabdoid phenotype, and (e) large cell neuroendocrine carcinoma (Brambilla et al, 2001).

2. Biomarkers

2.1 Cancer biomarkers

Cancer biomarkers are evaluated for establishing disease predisposition, early detection, cancer staging, therapy selection, identifying whether or not a cancer is metastatic, therapy monitoring, assessing prognosis, and advances in the adjuvant setting.

There are several distinct types of biomarkers based on different areas: genetics, epigenetics, proteomics, metabolomics, imaging technology, and general physical techniques

(Sung and Cho, 2008). Genetics based cancer biomarkers utilize DNA arrays, polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), DNA sequencing, and fluorescence in situ hybridization (FISH) to detect the genetic alterations occurring in the cancerous state. Epigenetic modification usually occurs in CpG islands of the gene regulatory regions which results in gene silencing. These alterations can evade the cells from their normal cell cycle control and may result in cancer cells formation (Baylin and Chen, 2005; Belinsky, 2004). Protein techniques include mass spectrometry (MS), ELISA, and immunohistochemistry (IHC), which are utilized to discover novel cancer biomarkers, and the biomarkers are later validated in clinical trials. Metabolomics is concerned with the study of low molecular weight molecules or metabolites such as amino acids, peptides, lipids, and carbohydrates. Imaging techniques such as positron emission tomography (PET), Computed Tomographic (CT) scans and Magnetic Resonance Imaging (MRI) are still major tools of cancer diagnosis and have the distinct ability to localize the cancer that molecular based biomarkers cannot do (Sung and Cho, 2008).

2.2 Lung cancer and its biomarkers

In patients with suspected lung cancer, a clear and definite diagnosis is essential for the treatment strategy (Pio et al, 2010). Late diagnosis is a fundamental obstacle to improving lung cancer outcomes (Carney, 2002; Chute et al, 1999). Therefore, early detection of lung cancer is obviously the only way to improve the overall survival. Some diagnostic tools including CT scans, bronchoscopy, and sputum analysis are routinely used at clinics, but none of them turns out to be effective in early diagnosis of lung cancer. There were 512948 publications from 'Pubmed' associated with biomarkers, and 14123 publications were associated with lung cancer biomarkers till the end of 2010. Only p63 and CK5/6 are widely used as diagnostic markers for SCC of lung, while thyroid transcription factor-1 (TTF-1) together with CK7 is considered as a marker for lung ADC. In some cases, even in combination with these markers, SCC and ADC still could not be distinguished from each other. Given the fact that early detection of lung cancer at stage IA can raise the 5-year survival rate from the overall 15% to 80% (Mulshine, 2005), reliable biomarkers for early diagnosis and survival prediction are increasingly in demand (Minna and Mangelsdorf, 1997; Hirsch et al, 2002).

3. Complement system and regulation of complement activity

The complement system is an integral part of the innate immune system. It consists of a number of small proteins found in the blood, generally synthesized by the liver, and normally circulating as inactive precursors (pro-proteins). There are three biochemical pathways that activate the complement system: the classical pathway, the alternative pathway, and the lectin pathway. When the complement system is stimulated by one of several triggers, proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavage. The result of the activation cascade is massive amplification of the response and activation of the cell-killing membrane receptors. The complement system can be extremely effective in destroying pathogens, but can be equally damaging to self-tissue. Complement regulation occurs predominantly at two steps within the complement cascade, the level of the convertase enzymes and of the membrane attack complex (MAC) (Liszewski et al, 1996) (Fig. 1).

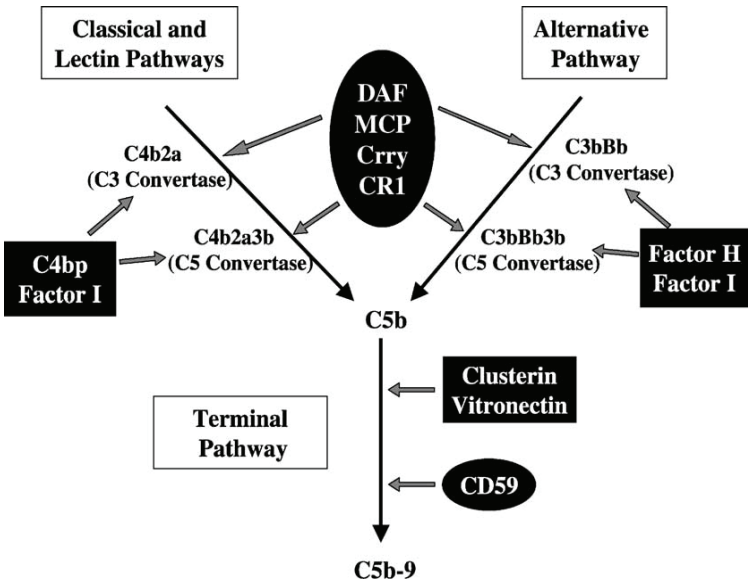

Fig.1 A schematic representation demonstrating that the complement regulatory proteins act either at the level of the C3/C5 convertase enzymes or within the terminal complement pathway. Membrane-bound factors are encircled whilst soluble regulatory proteins are boxed (Turnberg and Botto, 2003).

4. Complement factor H (CFH)

4.1 The CFH gene family and CFH structure

CFH is the best characterized protein of the CFH gene family, members of which belong to the regulators of complement activation family of proteins (Fig 2). CFH is a 154 kDa plasma protein, soluble glycoprotein that circulates in human plasma at a concentration of 235-810 µg/ml (Saunders et al, 2006). The family includes the complement regulators CFH and CFH-

like protein 1 (CFHL1), as well as five CFH-related proteins CFHR1-5 (Jozsi and Zipfel, 2008). CFHL1 proteins share complement regulatory functions with CFH and interact with heparin. The functions of CFH-related proteins (CFHR1 to CFHR5) are not well defined. CFHR1, CFHR2 and CFHR4 are constituents of lipoproteins, while CFHR3 is known to interact with heparin (Zipfel et al, 2002; Skerka et al, 1997; Hellwage et al, 1999).

CFH is made up of 20 complement control proteins (CCP) modules. Two major functional regions are located at the opposite ends of the protein. The N-terminal four SCR domains display complement regulatory activity by facilitating the decay of the C3 convertase and acting as a cofactor for factor I. The C-terminus of the protein mediates surface binding and target recognition (Oppermann et al, 2006)

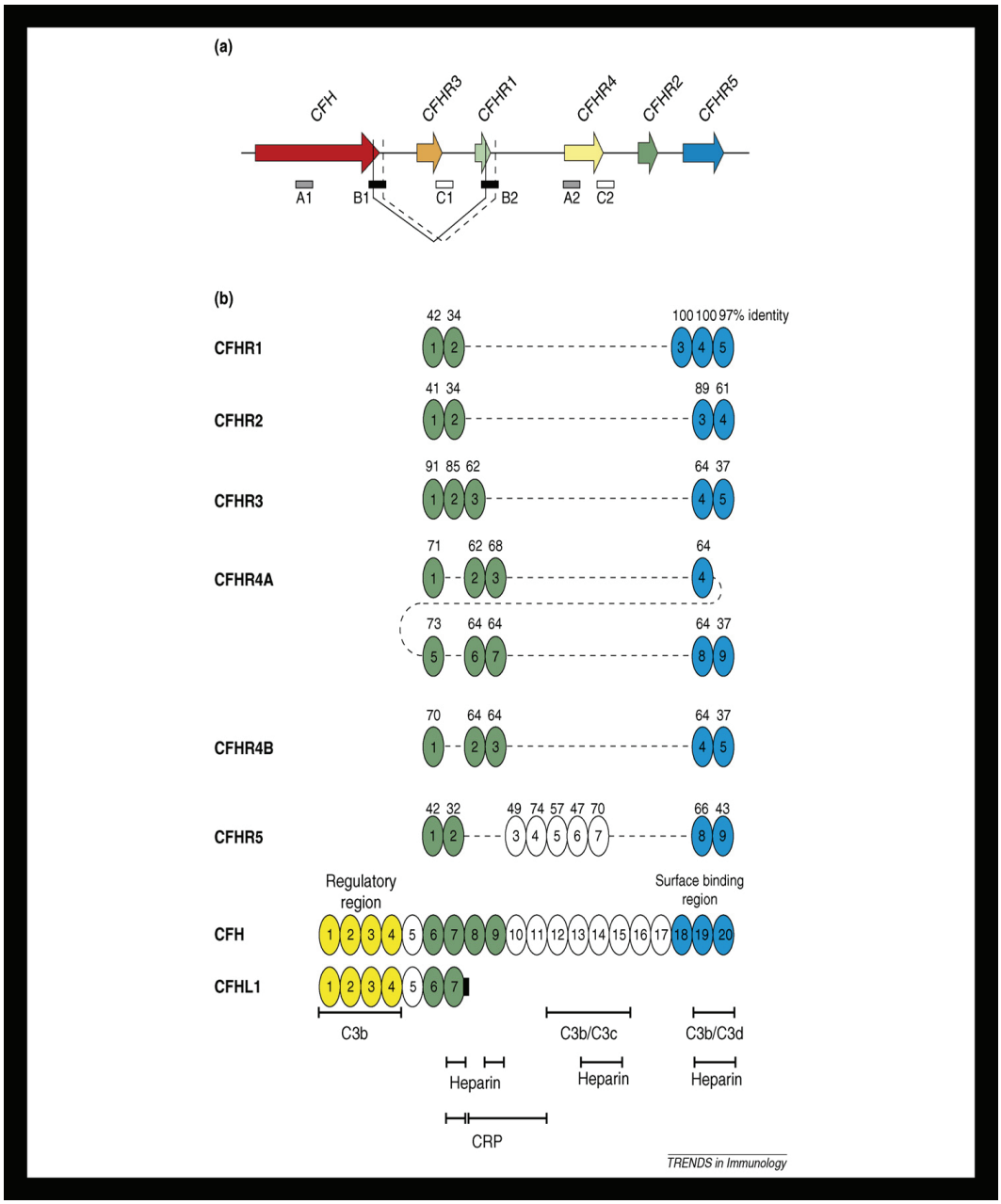

Fig. 2 The human complement Factor H gene cluster and structure of the various proteins

(a) The human factor H (CFH) gene cluster includes six genes that show a consecutive arrangement on human chromosome 1q32. This cluster spans a region of 415 kb and includes the CFH and five CFHR genes. For the CFH and the CFHR4 genes, two transcripts have been identified, which are derived by alternative splicing and which encode related but distinct proteins (CFH and CFHL1, as well as CFHR4A and CFHR4B, respectively). This cluster includes homologous repeat regions (marked as A1–C2), which can result in deletion of large chromosome fragment due to nonallelic homologous recombination events. Depending on the site of recombination as indicated by either the solid or the dotted lines, a large genomic deletion is observed in this cluster that either results in a CFH: CFHR1 hybrid gene (solid line) or the deletion of CFHR3 and CFHR1 (dotted line). These deletions predispose to atypical hemolytic uremic syndrome. (b) The CFH family proteins are plasma glycoproteins, being exclusively composed of short consensus repeat (SCR) domains, which are common among complement regulatory proteins. The individual SCR domains of the CFHR proteins share high sequence identity with each other and also with SCRs of the complement regulator CFH. Homologous domains identified by sequence similarity are indicated by vertical alignment, and the numbers above each SCR indicate the identity to the corresponding domain in CFH at the protein level. All CFHRs contain domains related to the C-terminal surface and ligand recognition region (SCRs 19–20) and to the middle region (SCRs 6–9) of CFH. CFHR proteins lack SCRs homologous to the complement regulatory domains (SCRs 1–4) of CFH. The conservation of the N and C termini among the CFHR proteins is indicative of related or even overlapping functions. For CFH, the localization of binding domains for C3b and its fragments, as well as for heparin and C-reactive protein (CRP), are indicated (Jozsi and Zipfel, 2008).

4.2 Function of CFH

CFH binds to C3b, accelerates the decay of the alternative pathway C3-convertase (C3bBb), and acts as a cofactor for the factor I-mediated proteolytic inactivation of C3b (Fig 3) (Rodriguez de Cordoba et al, 2004). Evidence supporting the involvement of CFH in carcinogenesis is accumulating. For instance, malignant glioblastoma cells produce CFH and CFHL-1 (Gasque et al, 1996; Junnikkala et al, 2000). Secretion of soluble complement inhibitor CFH and CFHL-1 by ovarian tumor cells could protect tumor cells against humoral immune attack and posed an obstacle for therapy with monoclonal antibodies (Junnikkala et al, 2002). Similarly, in colon and lung cancer, the possible role of CFH was resistance to complement attack (Wilczek et al, 2008; Ajona et al, 2004). Additionally, CFH or related

proteins were considered as diagnostic markers for transitional cell cancer of the bladder (Kinders et al, 1998).

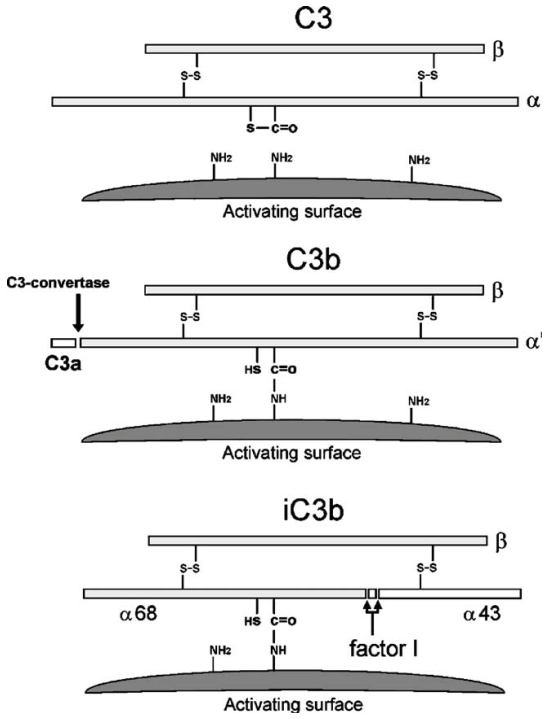

Fig. 3 Inactivation of C3b by factor H. Chain structure of the native C3, C3b and iC3b molecules. Sites of cleavage by the C3-converstase and factor I in α- and α'-chains of C3 and C3b are indicated by arrows. Inactivation of C3b to iC3b by factor I requires the cofactor function of factor H. The position of the internal thiolester that establishes the covalent linkage to the activating surface is also shown schematically (Rodriguez de Cordoba et al, 2004).

AIMS OF THE STUDY

The aims of the study were:

1. Investigate the expression and binding of CFH in human lung cancer cell lines
2. Analyze the location of CFH protein in human lung cancer cells
3. Explore the clinical utility of CFH in human lung cancer

PUBLICATION OVERVIEW

Human complement factor H is a novel diagnostic marker for lung adenocarcinoma

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DISCUSSION

The major findings present in the current study can be summarized as follows: 1) CFH was expressed in the majority of non-small cell lung cancer cell lines, 2) CFH was not expressed in small cell lung cancer cell lines, 3) cancer cells bound CFH to their surface, 4) the majority of tumor samples (53.5%) exhibited CFH positive staining, 5) CFH could be a biomarker for ADC of lung.

1. Expression of CFH in lung cancer cells

CFH is known as the most prominent fluid-phase complement inhibitor being present in plasma. It performs several activities which ultimately inhibit the alternative complement pathway. CFH was expressed in various malignant cell lines including ovarian, colon, bladder, liver, and lung cancer cell lines (Wilczek et al, 2008; Junnikkala et al, 2000; Cheng et al, 2005; Schlaf et al, 2002; Ajona et al, 2007). CFH binds to C3b and acts as a cofactor for the factor I-mediated proteolytic inactivation of C3b (Pangburn et al, 1977), while competing with factor B in binding to C3b to form the alternative pathway C3-convertase C3bBb (Whaley and Ruddy, 1976). It also accelerates the decay of C3bBb. By inhibiting alternative complement activity, factor H also protects tumor cells against immune attack (Rodriguez de Cordoba et al, 2004; Gasque et al, 1992). Earlier studies showed that, CFH is a marker for transitional cell cancer of the bladder (Kinders et al, 1998) and the utility of CFH as a diagnostic marker raised the question whether it could be a marker for other malignancies.

In our study, we analysed the mRNA and protein expression of CFH in a panel of lung cancer cell lines by RT-PCR, western blotting, flow cytometry, and immunofluorescence analysis. It turned out that several human NSCLC cells constitutively expressed and secreted CFH, while in SCLC cells, no CFH expression was detectable. These results are in line with previous report by Ajona et al (Ajona et al, 2004), who reported that neither SCLC nor carcinoid cell lines expressed CFH, suggesting a phenotypic correlation between the expression of CFH and the neuroendocrine differentiation of the tumors. We further analyzed the ability of factor H binding to lung cancer cells. It turned out that factor H binding to the cell surface is independent on its expression in tumor cells.

2. Expression of CFH in lung tumor tissues

In the survey of protein expression of CFH in 101 primary lung tumors, we found that more than half of the samples (53.5%) showed CFH positive staining, while the other tumor

samples were negatively stained, indicating that high expression of CFH is not an infrequent feature of this type of cancer. We could only divide the primary tumors into two major subgroups of ADC and SCC for statistical analysis, since the sample size of SCLC and LCLC was too small to be further evaluated. Although expression of CFH was not significantly associated with tumor progression, differentiation, and lymph node metastasis, it was significantly higher in ADCs (35/53) than in SCCs (16/41) ($p<0.01$). So far, the protein expression of CFH has only been evaluated in patients with primary colon adenocarcinoma by Wilczek et al. They found that CFH was expressed in both primary colon adenocarcinoma and metastatic foci in liver (Wilczek et al, 2008). It is not yet clear if overexpression of CFH is a common event in adenocarcinoma, nevertheless, the result suggests that CFH could be a novel marker to distinguish NSCLC from SCLC and may particularly identify lung adenocarcinoma.

3. Clinical usefulness of CFH in human lung cancer

It has been reported that some human NSCLC cell lines highly expressing CFH or CFH-like protein are more susceptible to complement–mediated damage and more efficiently deposited C3b on the cell surface when they are blocked by specific anti-factor H antibodies (Ajona et al, 2004). Tumor cells that express and bind CFH to their surface can prevent C3b accumulation upon their cell membranes leading to resistance of these cells to complement-mediated lysis (Ajona et al, 2004). Based on these characteristics, we supposed that tumors with high expression of CFH could be more aggressive and have a worse clinical outcome.

To test this hypothesis, we analysed the effect of CFH expression on patient survival. Again, in ADC of lung, there was a tendency that CFH positive-tumors had a worse clinical outcome compared to CFH negative-tumors, although it did not reach statistical significance. Interestingly, patients with ADC who had positive expression of CFH ended with a shorter survival time (less than 20 months). Evidence is not sufficient to prove the role of CFH as a prognostic marker in ADC of lung. Future studies with more tumor samples are needed to warrant the prognostic role of CFH in lung cancer; and we also plan to perform a prospective trial to detect the concentration of CFH in blood from lung cancer patients and healthy donors, to further investigate the clinical usefulness of CFH in human lung cancer.

Additionally, in line with the previous observations that CFH binds to apoptotic and necrotic cells (Leffler et al, 2010; Mihlan et al, 2009), we found that apoptotic cells expressed more CFH compared to non-apoptotic cells of primary lung cancer. Most apoptotic cells do not undergo complement-mediated lysis probably due to the overexpression of CFH, which

compensates the loss of m-C-Reg, a membrane-bound complement regulatory protein, and protect against excessive complement activation and lysis (Trouw et al, 2007).

Immunotherapy is an attractive approach that, by design, is cancer specific and can target disseminated disease with minimal impact on normal tissues (Hirschowitz and Yannelli, 2009). Immunotherapy is categorized as either passive or active. Passive immunotherapy includes any immunological active agent that is made outside the body and does not rely on host machinery to function. The most widely applied passive immunotherapies are monoclonal antibodies that disrupt tumorigenic cascades by blocking the binding of hormones or growth factors to their receptors; examples include cetuximab (Erbitux) and trastuzumab (Herceptin), which target epidermal growth factor (EGF) receptors; Gefitinib and Erlotinib, which are small molecular tyrosine kinase inhibitors, have directed activity toward EGFR; HER-1 and HER-2, respectively, and bevacizumab (Avastin), which interferes with tumor angiogenesis by binding to vascular endothelial growth factor (Sandler et al, 2004; Isobe et al, 2005; Giaccone, 2005; Baselga and Cortes, 2005; Baresi, 2010). By contrast, active immunotherapy uses the host's immune cells and requires an intact immune system to function. Active immunotherapy is derived from the knowledge that the immune system can discriminate cancer cells from normal cells based on tumor antigen recognition (Abu-Shakra et al, 2001; Pardoll, 2003; Raez et al, 2005; Novellino et al, 2005).

For long, lung cancer was not considered an immune-sensitive malignancy. Although there is increasing evidence that NSCLC and SCLC can evoke specific humeral and cellular anti-tumor immune responses, lung cancer immunotherapy lags behind similar efforts in melanoma, renal cell, and prostate cancer. Our results could provide further insight into the understanding of pathogenesis and could have implication for the design of more effective immunotherapeutic strategies for this fatal disease. For the next step, we plan to detect the concentration of CFH in blood from healthy donors and lung cancer patients (with NSCLC or SCLC, before and after chemoradiotherapy), to further investigate the clinical usefulness of CFH in human lung cancer.

In summary, non-small cell lung cancer expressed CFH, and CFH might be a diagnostic marker for ADC of lung.

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Publications:

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STATEMENT

I am familiar with the Promotionsordnung of the Faculty of Medicin of the University of Jena. All parts of the dissertation were produced by myself. I hereby declare that this thesis does not contain any material previously submitted for a degree or diploma at another university or any material previously written or published by any other person, except where due acknowledgment or reference is made in the text. I also declare that I did not obtain the assistance of a dissertation counseling agent and that I did not provide any direct or indirect financial remuneration to any third party in connection with the content of my dissertation.

Jena, 01. March. 2011 …….

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Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

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mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben:

- Prof. Dr. Iver Petersen

- Dr. Yuan Chen

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

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