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Role of Frizzled6 in the molecular mechanism of beta-carotene action in the lung

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ABSTRACT

β-Carotene (BC) is omnipresent in our diet, both as natural food component as well as an additive. BC and its metabolites have important biological functions. For this reason, BC is generally considered to be a health promoting compound. Two human trials, however, have described adverse effects in lung tissue, increasing the risk of lung cancer. We previously applied transcriptomic analyses in a unique animal model, beta-carotene 15,15′-monooxygenase 1 knockout (*Bcmo1*$^{-/-}$) mice that are, like humans, able to accumulate intact BC. In our search to unravel the molecular action of BC in the lung, we previously identified two genes particularly strongly down-regulated by BC in lung tissue of the male *Bcmo1*$^{-/-}$ mice: frizzled homologue 6 (*Fzd6*) and collagen triple helix repeat containing 1 (*Cthrc1*). In the present study, our aim was to further elucidate the role of *FZD6* in lung epithelial cells and to provide a mechanistic explanation for BC increased lung cancer risk in humans. We performed whole genome microarray analysis on silenced *FZD6* in non-tumor human type II bronchial epithelial BEAS-2B cells using RNAi. To directly link *FZD6* to BC-effects on the lung, we compared the *FZD6*-silenced BEAS-2B gene expression profile to the BC-dependent gene expression profile of *Bcmo1*$^{-/-}$ mouse lungs. A number of relevant genes were regulated in the same direction in *FZD6*$^{-}$ BEAS-2B and in BC-exposed lungs of *Bcmo1*$^{-/-}$ mice and revealed enrichment of the Gene Ontology terms "oncogenes", "cell proliferation" and "cell cycle", which suggests a mediating role of *FZD6* in BC-induced uncontrolled proliferation of lung cells.

1. Introduction

An on-going controversy exists on beneficial *versus* harmful effects of high dosages of beta-carotene (BC) on the development of lung cancer (van Poppel, 1996; Ziegler, 1989). According the WHO, lung cancer has been the most common cancer for decades and still has the highest prevalence worldwide (WHO, 2011). BC has largely been demonstrated to be a health-promoting agent. Indeed, many epidemiological studies showed that an increased intake of dietary BC, as well as increased BC plasma concentration, is associated with a decreased risk for cardiovascular diseases and several types of cancer, including lung cancer (van Poppel, 1996; Ziegler, 1989). However, convincing evidence from two large human intervention trials in at-risk individuals has shown that BC supplements increased the risk of lung cancer (Albanes et al., 1996; Omenn et al., 1996). Although these data question the safe use of BC at high levels of intake, BC supplements remain popular because of its attributed health-promoting properties. Moreover, BC is an abundant dietary constituent, and a ubiquitous additive.

BC and its primary metabolite retinol (vitamin A) are necessary dietary constituents, since humans are unable to synthesize them. Beta-carotene 15,15′-monooxygenase 1 (BCMO1) is a key

Abbreviations: BC, β-carotene; BCMO1, beta-carotene 15,15′-monooxygenase 1; Fzd6, frizzled homologue 6; Cthrc1, collagen triple helix repeat containing 1; GO, Gene Ontology; DAVID, Database for Annotation Visualization and Integrated Discovery; DO, Disease Ontology; FunDO, Functional Disease Ontology Annotations; ES, Enrichment Score; FDR, Benjamini–Hochberg false discovery rate; ATF2, Activating transcription factor 2; JUN, Jun oncogeneJUN; RHOA, Ras homolog gene family member A; RAC1, Ras-related C3 botulinum toxin substrate 1; CDC42, Cell division cycle 42; DVL1, Dishevelled segment polarity protein 1; MAPK9, Mitogen-activated protein kinase 9 (also known as JNK2); PARD6A, Par-6 partitioning defective 6 homolog alpha; FOXM1, Forkhead box M1; CENPE, Centromere associated protein E; KIF11, Kinesin family member 11; CDCA8, Cell division cycle associated 8; TFCP2, Transcription factor CP2; IRX4, Iroquois related homeobox 4; AEBP1, AE binding protein 1; HCC, hepatocellular carcinoma.

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enzyme in BC metabolism. It cleaves BC symmetrically to form two molecules of retinal, which can be further metabolized into retinol or several other downstream BC metabolites (von Lintig and Vogt, 2000). In humans a significant portion of BC is found intact in plasma, while rodents cleave virtually all absorbed BC because they possess a more active BCMO1 variant than humans (von Lintig and Wyss, 2001). In our search to unravel the molecular action of BC in the lung, we showed that the lungs of male *Bcmo1*$^{-/-}$ mice, which resemble the human condition, showed a marked and highly significant decrease of two genes, frizzled homologue 6 (*Fzd6)* and collagen triple helix repeat containing 1 *(Cthrc1*), when fed with BC (van Helden et al., 2010a,b).

FZD6 is a member of the frizzled family of conserved transmembrane receptors mediating WNT signaling (Tokuhara et al., 1998). In mammals, *FZD6* controls a wide variety of development-related processes, such as midbrain morphogenesis, neuronal tube, platelet and hair pattern development, and it has a role in controlling nail dysplasia and planar polarity of inner-ear sensory hair cells (Guo et al., 2004; Wang et al., 2010; Tsai et al., 2006). However, *FZD6* primarily functions in determining cell polarity and planar cell polarity signaling acting as a negative regulator of the canonical WNT pathway, which promotes cell proliferation (Golan et al., 2004; Devenport et al., 2011). Planar cell polarity is one of the several non-canonical pathways where different FZD-WNT signaling complexes act *via* a variety of downstream pathways and effectors (Schulte, 2010). *FZD6* might have an important function in the lung since it is highly expressed in adult and fetal human lung tissue, but the precise functions of *FZD6* in the lung are, as yet, unknown (Tokuhara et al., 1998). *FZD6* down-regulation after BC exposure may be a key factor in the molecular mechanism of BC-induced lung cancer development, but faithful interpretation requires understanding of molecular and functional consequences of *FZD6* down-regulation in lung cells.

Recent data has revealed that WNT pathways leads to tumor formation when aberrantly activated, through the control of the transcription of a suite of genes promoting cellular proliferation during carcinogenesis (Giles et al., 2003). Since FZD6 is expressed in gastric cancer and belongs to WNT pathway, a further analysis of FZD6, FZD6 target genes and genes involved in WNT non-canonical pathways may represent a valid tool to understand the mechanism of BC increasing lung cancer (Katoh, 2005).

The aim of our study was to investigate if down-regulation of *FZD6* in BEAS-2B cells would affect genes that were differentially expressed by BC in the lungs of male *Bcmo1*$^{-/-}$ mice. BEAS-2B cells are immortalized human lung cells with type II bronchial epithelial characteristics and are considered a good model for normal lung cells and an advantage in the investigation of carcinogenic processes (Garcia-Canton et al., 2013; Sun et al., 2011). Since BEAS-2B express *FZD6* at appreciable levels, but hardly express *CTHRC1*, this allowed us to investigate the consequences of down-regulation of *FZD6* in the context of low levels of *CTHRC1*, mimicking the mouse condition after BC treatment. RNAi was used to inactivate *FZD6*, whole genome microarray analysis was performed on *FZD6*-positive and *FZD6*-negative BEAS-2B cells and microarray data comparison between lung of *Bcmo1*$^{-/-}$ mice and *FZD6*$^{-}$ BEAS-2B cells was performed. This will help to elucidate the role of *FZD6* in lung cells and may contribute to a mechanistic explanation for the observed increased lung cancer risk by BC. This is necessary to establish unequivocal guidelines for a safe use of BC.

2. Materials and methods

2.1. Cell line

Human bronchial epithelial cells BEAS-2B were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and were cultured as prescribed by ATCC.

2.2. FZD6 *depletion using small interfering RNA (siRNA)*

RNA interference involves multiple RNA-protein interactions characterized by four major steps: assembly of siRNA with the RNA-induced silencing complex (RISC), activation of the RISC, target recognition and target cleavage. Target sites with low GC content have a greater potential for being a functional siRNA site, whereas a too high GC content can impede the loading of siRNAs into RISC complex. On the other hand, too low GC content can destabilize the siRNA duplex and reduce their affinity to target mRNA binding. For a better rate of success of a silencing experiment is therefore recommended to use both high and low GC content siRNA to evaluate which of the siRNAs provides the highest efficiency for the selected target gene (Amarzguioui and Prydz, 2004; Chan et al., 2009; Reynolds et al., 2004). Two specific *FZD6* siRNAs, named FZD6HSS112156 and FZD6HSS112157 that are complementary to different *FZD6* mRNA regions and contain high GC% and low GC%, respectively, and their correspondent scrambled negative controls, named HI GC and LO GC (all from Invitrogen, Breda, The Netherlands), were used separately to silence the *FZD6* gene. The fluorescently labeled siRNA negative control duplex AllStars Alexa 488 (Qiagen, Venlo, The Netherlands) was used to monitor the efficiency of transfection. SiRNA was transfected into BEAS-2B cells using Lipofectamine RNAiMAX (Invitrogen) and incubated for 48 hours according to the manufacturer. Cell viability was determined using Propidium Iodide (Invitrogen). Efficiency and viability were analyzed on Beckman Coulter Epics XL Flow Cytometer (FACS) with Expo32 software (both BD Biosciences, Erembodegem-Aalst, Belgium) according to Invitrogen and Qiagen protocols. Silencing was validated by real-time quantitative polymerase chain reaction (qRT-PCR) for the *FZD6* gene. A total of six samples with efficiency $\geq$85%, viability $\geq$85%, and with a knockdown of *FZD6* $\geq$94% and their correspondent scrambled negative controls were used for microarray analysis.

2.3. RNA isolation

Total RNA was isolated using TRIzol reagent (Invitrogen) followed by purification using RNeasy columns (Qiagen) according to instructions of the manufacturer. RNA concentration and purity were measured using the Nanodrop system (IsoGen Life Science, Maarssen, The Netherlands). Approximately 4 μg of total RNA was isolated from each sample with A260:A280 ratios >2 and A260:A230 ratios >1.9 for all samples, indicating good RNA purity. RNA degradation was checked on the Experion (Bio-Rad, Veenendaal, The Netherlands) using Experion StdSense chips (Bio-Rad).

2.4. Real-time quantitative polymerase chain reaction (qRT-PCR)

Silencing of the *FZD6* was validated by qRT-PCR. Preparation of the sample, concentration used, qRT-PCR detailed protocol, and calculations were carried out as described previously (van Helden et al., 2010a). Primers used are listed on Supplementary Table S1.

Supplementary table related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2014.03.002.

2.5. Microarray hybridization procedure

The 4 × 44k Agilent whole human genome microarrays (G4845A; Agilent Technologies, Santa Clara, CA, USA) were used. Preparation of the sample and the microarray hybridization were carried out according to the manufacturer's protocol with a few exceptions as described previously (van Helden et al., 2010a; Rodenburg et al., 2008; van Schothorst et al., 2007). Arrays were scanned with an Agilent scanner with 10% and 100% laser power intensities (Agilent Technologies). Feature extraction, quality control and normalization were carried out as described previously (van Helden et al., 2010a).

2.6. Data analyses of microarray results

Direct biological interaction analysis on the $P < 0.05$ gene list was performed as follows: Gene Ontology (GO) overrepresentation analysis by using the Database for Annotation Visualization and Integrated Discovery (DAVID) (Huang et al., 2009). Disease Ontology (DO) annotation to study gene-disease relationships by using Functional Disease Ontology Annotations (FunDO) and literature data mining (Osborne et al., 2009).

2.7. Comparison of human BEAS-2B lung cells FZD6-dependent gene expression with in vivo mice lung BC induced differential gene expression

The *in vivo* BC study was performed with mice, while the *in vitro FZD6* silencing experiment was performed with human BEAS-2B cells (van Helden et al., 2010a,b). To allow microarray data comparison of these two studies, the human homologue of the mouse geneIDs was retrieved *via* the NCBI homologene using our custom script in R (Pronk et al., 2012). The output was manually checked for missing geneIDs and duplicates, which were subsequently included *via* manual transfer of the data. Next, GeneID to symbol conversion was performed using the tools on the DAVID website (Huang et al., 2009).