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Review

Beta-carotene and the application of transcriptomics in risk–benefit evaluation of natural dietary components

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Abstract

Beta-carotene is a natural food component that is present in fruits and vegetables and is also used as a food colorant and a supplement. Beta-carotene is an anti-oxidant and a source of vitamin A. It is endowed with health beneficial properties, but a number of studies showed that with high intakes it may increase the risk for lung cancer in at risk individuals (heavy smokers, asbestos workers and alcohol users). To establish the window of benefit, it is necessary to identify early markers of effect and to obtain insight in the mechanism of action of beta-carotene, in the absence and presence of environmental risk factors. Genomics technologies are well suited to dissect the mechanisms of action and identify the markers of effect. Human cell lines can be used to analyse the effects of beta-carotene, but exposure studies with beta-carotene show that cell lines display a widely variant behaviour, which hampers translation to the in vivo situation in humans. Alternatively, animal studies can be used. Especially the ferret seems to be a good model, but little sequence information of this species is available. However, heterologous hybridization on human cDNA seems possible and provides and a new tool for molecular analysis of health effects of beta-carotene.
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Keywords: Beta-carotene; Microarray; Ferret; Cell line; Lung; Colon

1. Introduction

Beta-carotene is a natural compound that is omnipresent in yellow and green vegetables and fruits, such as carrot, spinach, sweet potato, pumpkin, broccoli, peppers, pink grapefruit, papaya and peach. Beta-carotene is also a colouring agent that is ubiquitously added to food and drinks. Furthermore, beta-carotene is an important source of vitamin A, since it can be into vitamin A upon metabolism in the body (Fig. 1). Beta-carotene is added to plant based margarines, to compensate for their lack of vitamin A. More recently, a rice variety (golden rice) has been made by genetic engineering that contains beta-carotene to combat vitamin A deficiency in developing countries. From the above it can be seen that beta-carotene is widely present in the diet and that it may influence our health. For this, it is important that upper and lower concentrations that define a minimum dose of necessary intake and a maximum dose of safe intake are properly defined. These concentration borders constitute the 'window of benefit', wherein a negative effect on our health is absent and a positive effect on our health can be expected. This review summarises the health beneficial and potential adverse effects of beta-carotene and describes how experimental research that makes use of genomics technologies can contribute to the understanding of the mechanism of action of beta-carotene. Understanding that is necessary to identify the early markers of effect and to define the window of benefit.

2. Beta-carotene is healthy

Beta-carotene intake levels vary substantially between people and countries. In a 5-country study [1], the median

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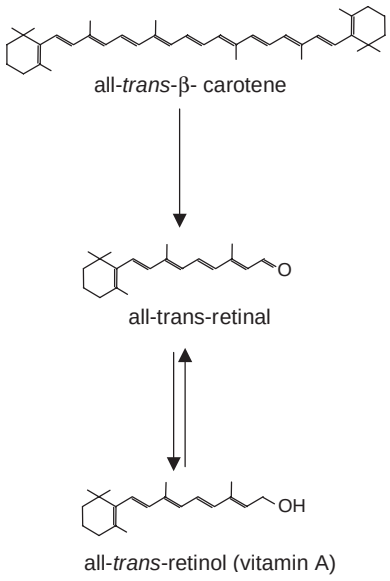

Fig. 1. Conversion of beta-carotene to retinol (vitamin A). In vivo beta-carotene can be converted to retinol. The most important route is thought to be the central cleavage of beta-carotene by beta-carotene-15,15′-dioxygenase, resulting in two molecules retinal. Subsequently, retinal reductase can reduce retinal to retinol.

intake of beta-carotene by adults varied from 2.96 mg/day in Spain to 5.84 mg/day in France. In the 5 countries, the following intake ranges were observed: 1.6–4.4 mg/day in Spain, 3.8–8.0 mg/day in France, 3.6–6.6 mg/day in the UK, 3.5–7.4 mg/day in Ireland and 2.9–5.7 mg/day in the Netherlands. The variation in intake was mainly due to differences in fruit and vegetable consumption. This is supported by other studies in other countries (e.g. [2–4]). The intake of beta carotene by food consumption results in adult human plasma beta-carotene levels of 0.2–0.5 μmol/l [5–8]. Several observational studies and case-control studies show that high intakes of carotenoid-rich fruits and vegetables, as well as high serum beta-carotene levels, are associated with a reduced risk for cardiovascular disease and a reduced risk for developing cancer, in particular of the stomach, oesophagus, lung, oral cavity, pharynx, pancreas and colon [9,10]. These observations endowed beta-carotene with health beneficial properties, which were explained by its anti-oxidant nature [11,12].

3. Doubts on beta-carotene

To prove that beta-carotene could reduce the incidence of cancer, in particular lung cancer, a double-blind, placebo-controlled trial, the Alpha-Tocopherol-Beta-Carotene (ATBC) trial [13], was conducted with 29,133 smokers between 50 and 69 years of age. The participants to this large chemoprevention study obtained a daily supplement that contained either 20 mg of beta-carotene or 50 mg of vitamin E, another powerful anti-oxidant, a combination of both or a placebo, for 5 to 8 years. This amounted to 10 times the median intake of beta-carotene and 5 times the median intake of vitamin E, also known as alpha-tocopherol. Unexpectedly, participants receiving beta-carotene, alone or in combination, had significantly higher lung cancer incidence (RR 1.16; CI=1.02–1.33) and higher mortality (RR 1.08; CI=1.01–1.16) than did the subjects receiving the placebo. Subgroup analysis revealed a higher risk in heavy smokers (20 or more cigarettes/day) (RR 1.25, CI=1.07–1.46) as compared to light smokers (5–19 cigarettes/day) (RR 0.97, CI=0.76–1.23). Interestingly, both dietary intake and serum beta-carotene levels at the start of the trial were found to be inversely related to the risk of lung cancer during the trial. No evidence of an interaction of beta-carotene and vitamin E was found [13]. Besides lung cancer, also the mortality from cardiovascular disease appeared to be increased by beta-carotene [14].

In a second chemoprevention trial, the Beta-Carotene Retinol Efficacy Trial (CARET), similar results were obtained. In this study 18,314 participants (14,254 male and female (45%) smokers and former smokers between 50 and 59 years of age and 4060 asbestos-exposed males between 45 and 74 years of age) were given a daily supplement of 30 mg beta-carotene and 25,000 IU vitamin A (retinyl palmitate). Also here, an increased risk was found for lung cancer incidence (RR of 1.28, 95% CI=1.07–1.57) and lung cancer mortality (RR of 1.46; 95% CI=1.13–2.00) for the exposed group compared with the placebo group [15]. The CARET Study also showed an increased number of deaths from cardiovascular disease in the group receiving beta-carotene and retinyl palmitate. The results of both trials came as a surprise. The outcomes are best explained by the combination of the high dose of beta-carotene and the fact that the participants were at high risk for developing lung cancer. They were former and present smokers and asbestos-exposed workers. In two other trials with mainly non-smokers, beta-carotene had no effect [8,16]. Similar results were described in a study by Baron et al. [17], which focussed on colon adenoma recurrence. These authors observed that 25 mg/day beta-carotene decreased the risk for adenoma recurrence in participants who neither smoked cigarettes nor drank alcohol (RR=0.56, CI=0.35 to 0.89), but a mildly increased risk was observed in smokers as well in alcohol drinkers. For participants who smoked and drank more than one alcoholic drink per day a risk doubling was observed (RR=2.07, CI=1.39 to 3.08; P for difference from nonsmoker/nondrinker RR<0.001). Although there is no evidence of adverse effects of beta-carotene at normal or slightly elevated levels of intake, the outcome of especially the ATBC and CARET studies resulted in the conclusion of the EU scientific committee on Food [18] that supplemental beta-carotene, at 20 mg/day or more, is contra-indicated for use in current, heavy smokers. This committee also concluded that there is

insufficient scientific basis to set a precise figure for an upper limit of isolated beta-carotene.

4. Upper limits

To understand why it is difficult to set upper limits, in particular for natural dietary constituents, it is necessary to have some insight in the process of risk assessment. This process consists of the following steps. First, potential harmful components and conditions are identified. Second, the level of exposure (intake) is assessed. This may differ substantially for different subgroups such as children or preferred intake groups. Third, the hazard is characterized. In other words, effects are measured. Finally, the risk is characterized by the integration of all available information and recommendations are made for levels of intake.

Food safety assessment is in fact food chemical safety assessment, where the safety of an individual chemical that is present in our diet or food is assessed. This is generally done using a pharmacological approach, where individual components are tested and the potential risk is assessed. In this approach animals are exposed to different concentrations of a food chemical. High doses are used in order to see the effects in limited numbers of animals. From a dose-effect curve, a No Adverse Effect Level is determined. Uncertainty factors are applied to correct for species differences, uncertainty and differences in susceptibility and an acceptable daily intake is derived (Fig. 2). This process works extremely well to ensure the safety of our food and protects us from the deleterious effects of exogenous chemicals. An important question is how this will work for components that are naturally present in our diet, especially since large numbers of natural components would be 'toxic' when tested at 100 times their natural dose [19]. One of the reasons for this apparent discrepancy is that screening assays do not faithfully predict risk or benefit, but assess the potential risk. Furthermore, individual components are tested using relative short exposure times and high concentrations. This starkly contrasts to dietary exposure, where individuals are exposed to natural food components as a complex mixture, during a lifetime, and risks and benefits will be determined by relative small differences in the concentration of the different components. Consequently,

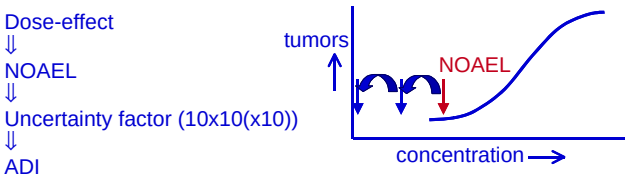

Fig. 2. Determination of the acceptable daily intake (ADI). The ADI is derived from a dose-response curve, that is made in an animal exposure study. From this curve a No Adverse Effect Level (NOAEL) is determined. After the application of uncertainty factors an ADI is formulated.

the question of risks and benefits of natural bioactive food components can be translated into: what are the effects of chronic exposure at relevant doses of intake.

5. Risk–benefit assessment and functional genomics

To efficiently determine the effects on our health of chronic exposure at relevant doses of intake, sensitive markers are necessary that can identify long-term health effects at an early stage. Such markers are lacking at this moment and the assessment of benefits and risks is principally based on the association with outcomes that occur over a lifetime, such as ageing, diabetes type II, cancer and cardiovascular disease, or are at best intermediate, such as cholesterol levels. How can we identify good, sensitive, early markers that strongly correlate with established health parameters? In other words how can we identify early changes that can be used as predictive markers. A technological solution is the use of functional genomics. Functional genomics studies populations of molecules, mRNAs, proteins or metabolites, rather than individual molecules, and as such a spectre of effects can be detected. The large number of parameters that can be analysed at the same time not only enhances the chance of finding something, but also increases power, since it allows the use of multiple small effects, if these effects are connected. For these reasons functional genomics technologies, collectively known as 'omics', are well suited to dissect working mechanism at the molecular level. Here we will focus on analysis of changes in cellular mRNA populations, which is named transcriptomics ([20,21], Fig. 3), but similarly proteins, using proteomics [22], or metabolites, using metabolomics [23], can be analysed. Proteomic analysis has the advantage that it directly analyses the protein, which is the metabolically active gene product, but has the disadvantage that a smaller number of parameters can be analysed at the same time. Metabolomics is even more under development and seems best suited to identify the biomarkers of exposure rather than to dissect biochemical mechanisms.

Transcriptomics is done using DNA microarrays. These are glass slides containing different cDNAs or oligonucleotides at fixed positions, each representing a different gene. The mRNA population that is assessed is isolated as an RNA pool, labelled with a fluorescent dye and hybridized to the glass array. After washing and scanning, the amount of fluorescence present at each position corresponds to the amount of a specific mRNA that was present in the original sample. This amount represents the transcriptional activity of the corresponding gene. DNA microarrays can be used to simultaneously analyse the response of thousands of genes to exposure to different levels of, for example, beta-carotene. Data analysis using bioinformatical and statistical tools allows the identification of molecular and physiological pathways that are affected.

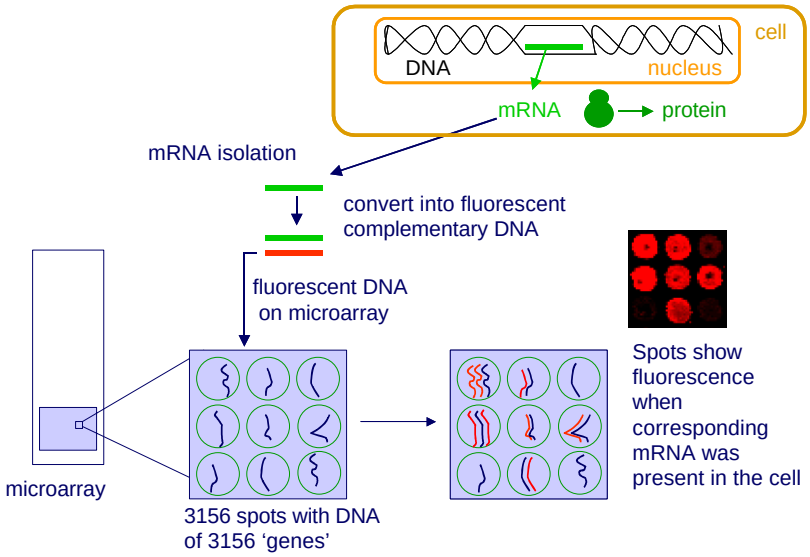

Fig. 3. Transciptomics. For transcriptomics analysis, mRNA is isolated from a cell or tissue, converted into fluorescent cDNA and brought onto a DNA microarray. This is a glass slide with DNA sequences in specific positions, each representing a different gene. Each fluorescent cDNA binds specifically to the complementary sequence and the amount of fluorescence in each position is a measure for the transcriptional activity of the corresponding gene.

This provides a starting point to understanding the molecular effects of exposure to different levels of bioactive food components and thus to the identification of sensitive, early biomarkers.

6. In vitro studies

To study the effects of beta-carotene, humans, animals and cell cultures can be used. An analysis of the molecular effects of beta-carotene on the human lung is not possible due to limitations in obtaining samples. This may change in the (distant) future if nanotechnological sample procedures will become operational and the sensitivity of the analytical techniques will have increased. Animal studies provide an alternative, although care needs to be taken to validate the model relative to the human. Human cells grown in tissue culture dishes, in vitro studies, provide another alternative and have the advantage that such studies are relatively easy to perform. An important aspect of in vitro studies is to consider how the natural delivery of beta-carotene to cell cultures can be mimicked. In the intact organism, beta-carotene uptake proceeds as follows. In the lumen of the small intestine beta-carotene is incorporated in lipid micelles. Taken up in the intestinal cells, beta-carotene is incorporated in chylomicrons and excreted into the lymph. Next, it is taken up in the liver and excreted as lipoprotein particles into the circulation to arrive at the different tissues of the body. Natural exposure is thus best mimicked by lipid micelles for intestinal cells, by chylomycrons for liver and by lipoprotein particles for all other tissues. In practice it is virtually impossible to obtain beta-carotene loaded lipoprotein particles in a reproducible manner. Therefore, in most cases beta-carotene is delivered in a solvent (e.g. tetrahydrofurane, THF) to the serum of the culture media. Alternative options are liposome delivery and detergent (Tween 40/80) mediated delivery, which is used by a limited number of scientists. We have used a mixture of linoleic acid (LA) and beta-carotene and thus deliver beta-carotene in a lipid suspension, resembling micelles. This allowed us to use THF, which was necessary for dissolving the beta-carotene, at very low concentrations (0.02%), thus preventing the toxic effects of the solvent as observed at 0.5% ([24], Dembinska-Kiec et al., personal communication). Before we started to analyse the molecular effects of beta-carotene exposure, we decided to first determine the uptake of beta-carotene. We examined four human intestinal cell lines (two colon derived tumour cell lines, CaCo-2, HT-29 and HT29D4, the SV-40 immortalized colon cell line CCD841CoTr and the small intestinal cell line HuTu 80) and four human lung cell lines (the lung tumour derived cell lines NCI-H292, NCI-H460, NCI-H661 and the SV-40 immortalized cell line BEAS-2B), expecting to find similar uptakes within both groups of cell types. To our surprise, this was not the case [24]. Both kinetics and levels of uptake (Table 1) were different for each cell line. Furthermore, no specific lung or intestinal profile could be detected. Since very little information is available on the uptake and metabolism of beta-carotene in vivo in human lung and intestinal epithelial cells, it is not possible at this moment to conclude which cell line represents the in vivo situation best. Our results show that in using in vitro cell systems extreme care has to be taken not to interpret results based on a single cell line towards an effect on our health, without confirming that the same process takes place in the same manner in an intact human individual.

Despite these reservations, we investigated whether exposure to beta-carotene would result in molecular changes

Table 1
Steady state intracellular beta-carotene

Cell line	Concentration (μM)
CaCo-2	2.7
HT-29	1.5
HT29D4	4.8
CCD841CoTr	17.7
HuTu 80	11.8
NCI-H292	54.7
NCI-H460	9.4
NCI-H661	35.0
BEAS-2B	12.6

Intracellular beta-carotene concentrations were determined based on HPLC determined beta-carotene concentrations per million cells and on cell diameters as measured in coulter counter, assuming that the cells were spherical. Cells were exposed to 1 μM beta-carotene (in 0.02% THF and 2.5 μM linoleic acid) in RPMI-1640 medium with 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (25 μg/ml), 1% non-essential amino acids at 3 °C, 5% CO_2, in a humidified atmosphere with daily refreshment of the medium. Intracellular beta-carotene concentrations are given at maximum values. This is the plateau value for most cell lines, which is reached after 56 h or earlier, except for CaCo-2 and HT-29 and HT29D4, where the concentration after 500 h is given.

in the high uptake lung cell lines BEAS-2B and H661. We anticipated that this could provide leads for detailed molecular studies in humans or animals. Cells were exposed to 1 μM of beta-carotene for 76 h with daily refreshment of the medium. Cells were harvested and RNA was isolated from exposed and non-exposed cells and queried using transciptomics. A cDNA microarray containing 3156 sequence verified, functionally annotated human cDNAs was used. The complete experiment was performed in 5-fold for H661 and 6-fold for BEAS-2B. As can be seen in the principal component analysis shown in Fig. 4A, the basal gene expression differences between the cell lines are bigger than the gene expression changes induced by beta carotene. Again confirming the differences between the cell lines. Beta-carotene exposure induces changes in overall gene expression (Fig. 4B). However, the magnitude of the changes between beta-carotene exposed and non-exposed cells was small, a maximum of 2.4-fold for H661 and 2.1-fold for BEAS-2B. This may indicate mild effects of beta-carotene, but can also be explained by the fact that effects were analysed under conditions of steady state (76 h). Further bioinformatical analysis of the in vitro studies may allow for the identification of differentially expressed genes and thus provide leads for in vivo studies.

7. In vivo studies

In parallel to the in vitro studies, we also proceeded with in vivo analysis, using laboratory animals. Rodents, which are the dominant species used in laboratory experiments, seem not to be a good model for studying the effects of beta-carotene. In rodents, beta-carotene is efficiently converted to retinal and almost no beta-carotene is observed in the circulation after dietary beta-carotene supplementation [25]. This contrasts with humans, where about 20–75% of the beta-carotene is absorbed intact [26,27]. The pre-ruminant calf [28] and the Mongolian gerbil [29] have been proposed as animal models for the study of beta-carotene absorption and metabolism in humans. Alternatively, ferrets have been proposed as a good model [30], because the ferret is most similar to humans in the (a) absorption of intact beta-carotene, (b) accumulation of beta-carotene in lung tissue,

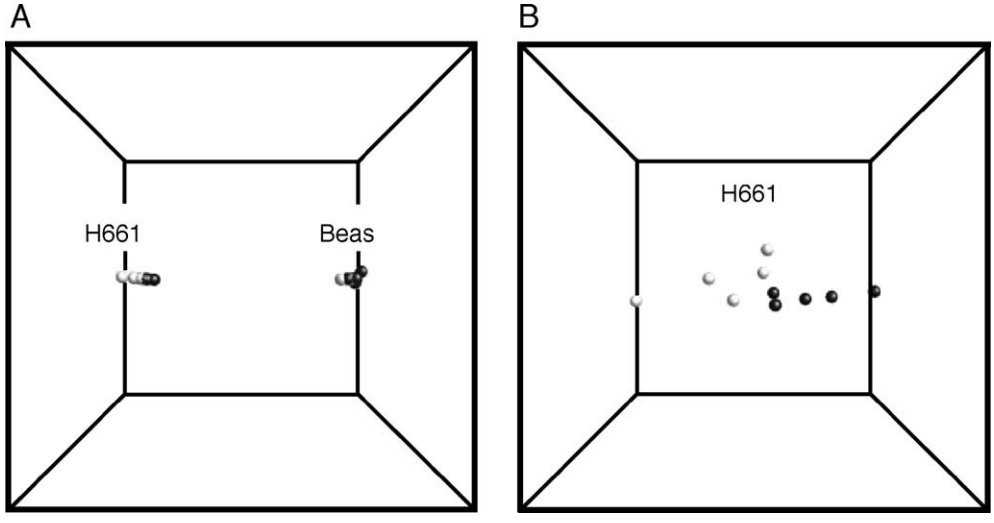

Fig. 4. Principal component analysis of overall gene expression differences between beta-carotene exposed and non-exposed human lung cell lines. The cell lines H661 (done in 5-fold) and BEAS-2B (done in 6-fold) were exposed to 1 μM beta-carotene (in 0.02% THF and 2.5 μM linoleic acid) in RPMI-1640 medium with 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (25 μg/ml), 1% non-essential amino acids at 37 °C, 5% CO_2, in a humidified atmosphere with daily refreshment of the medium for 76 h. Gene expression profiles of each exposure were independently determined [37], using a human cDNA microarray containing 3156 different human cDNAs. Variation in overall gene expression was analysed by Principal Component Analysis (PCA). Panel A shows the differences between exposed (light grey) and non-exposed (black) H661 and BEAS-2B cells. Panel B zooms in on the exposed (light grey) and non-exposed (black) H661 cells.

(c) appearance of oxidative metabolites of beta-carotene in the lung, (d) lung architecture and (e) lung pathology induced by cigarette smoke. The domestic ferret (*Mustela putorius furo*), which belongs to the same family as weasels and otters, lives 6–10 years and is sexually mature after one year. A typical female weighs up to 1.1 kg and is 33 to 35.5 cm long, while males weigh up to 2.7 kg and are 38 to 40.6 cm long. Wang et al. [26] used the ferret and proposed that beta-carotene supplementation resulted in lower levels of retinoic acid in the lung tissue, which may cause diminished retinoic signalling, enhanced lung cell proliferation and potential tumour formation. Alternative explanations for the adverse effects of beta-carotene supplementation on the lung of at risk individuals include the high oxygen pressure in the lung which may shift the antioxidant activity of carotenoids into pro-oxidant activity. Another explanation is that beta-carotene increases the formation of carcinogenic metabolites from cigarette smoke constituents, by the induction of the activity of P450 enzymes, in particular CYP 1A1 and 1A2 [31,32]. At this moment it is unclear what the molecular effects of beta-carotene are and which precise mechanisms underlie the adverse effects of beta-carotene in at risk individuals.

In the EU-funded DLARFID project, it was decided, based on the arguments given above, to use the ferret as a model to study the effects of beta-carotene and to specifically analyse the interaction between beta-carotene and benzo[a]-pyrene, a model carcinogen that is present in cigarette smoke. Performing functional genomic studies with the ferret poses a problem, since virtually no genomic information is available on this species. The paucity of sequence information is illustrated by the presence (August 2004) of only 45 nucleotide sequences and 47 protein sequences in the NCBI/EMBL/DBJ databases. Consequently, no ferret arrays are available for large-scale gene expression analysis. Therefore, in a pilot experiment we have tested whether cDNAs arrays from other species can be used. We hybridized ferret RNA on mouse and human arrays with over 3000 different cDNAs. The hybridization of 3 different ferret samples showed an average of 32% of the positions with a signal of 3 times above the background on the human array (an example is shown in Fig. 5), while this was 23% for the mouse cDNA array. Based on these results, it seems that a human cDNA array can be used to address the molecular effects of beta-carotene, although some information may be lost. Indeed the hybridization of human lung RNA showed

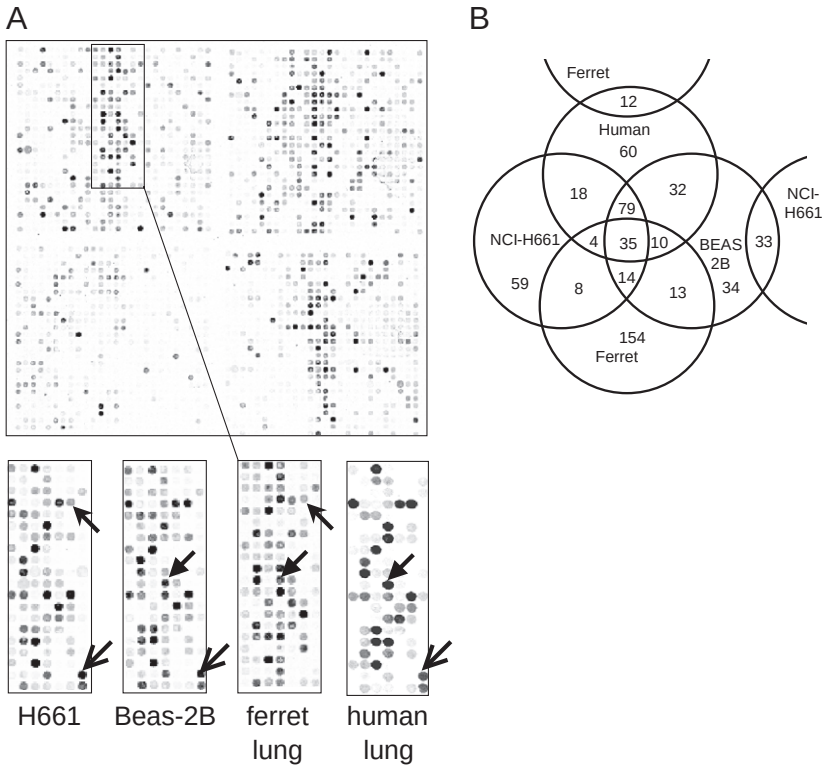

Fig. 5. Gene expression analysis of the ferret on a human cDNA microarray. Ferret lung and adipose tissue, human lung and human lung cell lines H661 and Beas-2B RNA was reverse transcribed and simultaneously labeled with a fluorescent dye. The labeled cDNA was hybridized to a 3154 feature human cDNA microarray and scanned in a laser scanner. (A) A representative part of the microarrays is shown. The dots represent the fluorescence signal at each position. A clear overall similarity between lung expression patterns can be seen. Arrows indicate differences and similarities between the lung samples. (B) (Dis)similarities between ferret lung, human lung, H661 and Beas-2B gene expression given as the number of 250 highest expressed genes occurring commonly or uniquely for the different samples. It should be kept in mind that due to lower overall hybridization of ferret lung, the figure represents the minimal amount of similarity for this sample.

56% of the positions with a signal above 3 times background and the beta-carotene exposure experiments of BEAS-2B and H661 showed 51% and 47% of the positions with a signal above 3 times background, using a similar human cDNA array. Although the number of hybridizing genes of the ferret is less, the expression pattern shows a clear visual similarity (Fig. 5A). An analysis of the 250 highest expressed genes on each array reveals that 61 (24.4%) of the highest expressed ferret genes are also among those of the human lung sample (Fig. 5B). The relatively low number can be due to differences between human and ferret, but can also be false negatives, which will occur due to low levels of sequence homology. The latter notion is supported by the observation that all of the 250 highest expressed ferret lung genes were found to be expressed above 4.5 times background in human lung and/or lung cell lines, with the expression of 2, 12 and 11 genes being absent above 4.5 times the background in the human lung, H661 and Beas-2B samples, respectively. This indicates that a larger than usual number of false positives is not anticipated. There is of course a need for caution concerning the absolute reliability of the data obtained when using heterologous RNA samples on the arrays and a confirmation of the microarray results using independent technologies will be necessary. For this, we can use Northern blotting or quantitative real time PCR [33] in the latter regions of homology between the same genes of a number of different species can facilitate primer development.

Genomic technologies will undoubtedly contribute to the understanding of the effects of nutrients and nutrition on our health [34–36]. It will also contribute significantly to our understanding of the mechanism of action of beta-carotene. Understanding that is necessary to identify early markers of effect. However, as can be seen from the examples provided, there are a number of hurdles to tackle before we will be able to accurately predict and to define the window of benefit of beta-carotene.

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