



Ketone bodies production and utilization by the two breast cancer compartments/ An immunohistochemical study in Kurdistan region-Iraq

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Abstract

Background Because of the close relationship between the cancer cells and cancer associated fibroblasts, it is increasingly clear that the development of cancer cannot be dissociated from its local microenvironment. Many previous researches were done to prove the role of cancer associated fibroblasts in fueling the cancer epithelial cells with ATP and lactic acid. **Objective** This study was designed to clarify the role of cancer associated fibroblasts in feeding the breast cancer epithelial cells with ketone bodies and indirectly to anticipate the efficacy of ketogenic diet in breast cancer patients. **Materials and methods** Forty selected cases of invasive breast carcinoma not otherwise specified (NOS) with a mean age of 47.83 ± 12.04 were included in this study and immunohistochemically stained with two mitochondrial enzymes antibodies: HMGCS2 and ACAT1; that play important roles in synthesis of the primary ketone body; acetoacetate and in ketone breakdown (ketolysis) during the fat processing respectively. **Result** Our result showed that both cell compartments harbor the enzymes needed for ketone bodies production and utilization. Cancer epithelial cells contain HMGCS2 and ACAT1 in 97.5% and 87.5% respectively. While cancer associated fibroblasts contain HMGCS2 and ACAT1 in 95% and 67.5% respectively. **Conclusion** both compartments can efficiently produce and utilize the ketone bodies, so indirectly we can say that ketogenic diet may have limited role in breast cancer management.

Introduction

Tumor microenvironment becomes a major title in upcoming and new researches, regarding how tumor cells get their energy fuel. Interestingly ketone bodies are oxidized via the Krebs cycle to provide the energy required by different tissues, most importantly, they can be utilized under conditions of hypoxia, when oxygen is scarce [1, 2]. Potentially, in case of a tumor mass this would allow a tumor to grow even in the absence of vascular supply. Thus, ketone body fuel may be important for initial tumor cell growth before the establishment of a vascular supply or for tumor spread [3-5]. The Warburg Effect which adopted by Otto Warburg in 1924, demonstrates that tumor cells change their metabolism in order to fuel their own growth [6]. The paradox is that cancer cells rely on glycolysis even if oxygen is available through production of lactic acid and ATP. This phenomenon is called aerobic glycolysis or Warburg effect [7, 8]. There are debates on this finding, more recent researches have been made and demonstrate that when cancer progresses, the increasing numbers of the stromal cells eat themselves to provide recycled nutrients in the form of lactic acid to tumor cells and this is named "The Reverse Warburg Effect"[9]. The reverse Warburg effect in human breast cancer was first proposed by Dr. Lisanti and colleagues in 2009. According to his model, aerobic glycolysis actually takes place in cancer associated fibroblasts (CAFs) and not in cancer cells.

So reverse Warburg effect reflects the role of the tumor microenvironment especially the CAFs in fueling the cancer epithelial cells with lactic acid and ATP [10-12]. In human beings and most other mammals, acetyl-CoA formed in the liver during oxidation of fatty acids may enter the citric acid cycle (Krebs cycle) or it may be converted to ketone bodies; “acetoacetate, D-β-hydroxybutyrate, and acetone” to be exported to other tissues [13]. Ketone bodies are used as energy fuels through conversion to Acetyl-CoA, which can then be used via oxidative mitochondrial metabolism to generate high amount of ATP [14].

In this study we have tried to illustrate the tumor ability for self-dependence on ketone bodies for fueling cancer epithelial cells produced from the CAFs through the expression of the two proteins; 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), which is a mitochondrial enzyme used in ketone bodies production [15] & acetyl coenzyme A transferase 1 (ACAT1), also known as thiolase; is a mitochondrial enzyme used in ketone bodies utilization [16] in two compartments of the breast cancer, including cancer epithelial cells and cancer associated fibroblasts. Aim of this study to highlight the expression of the mitochondrial enzymes in both breast cancer compartments and indirectly to anticipate the role of ketogenic diet in breast cancer patients.

Materials and methods

Study area

In this study forty female patients with breast invasive ductal carcinoma (NOS) were included; paraffin blocks were collected from Sulaimani teaching Hospital in Kurdistan region of Iraq. Re-sectioning, re-evaluation and immunohistochemically staining were done using two primary antibodies; ACAT1 (SC-161307) polyclonal antibody and HMGCS2 (SC-367092) monoclonal antibody by using the immunoCruz™ ABC detection kit at low pH antigen retrieval and at 1:100 antibodies dilution. Materials were purchased from Santa Cruz Biotechnology Inc., (U.S.A.). Kidney tissue was used as positive control for ACAT1 by illustration of cytoplasmic positivity in renal tubules, while liver tissue was used as the positive control for HMGSC2 through the staining of hepatocytes cytoplasm.

Immunohistochemical scoring

The intensity and percentage of staining were scored in cancer epithelial cells and CAFs as follow:

- The intensity was scored as 0 = negative or no stain, 1 = low, 2 = intermediate and 3 = high
- The percentage of stained cells was performed at 400x total magnification as: sporadic i.e. < 10%, focal i.e. $\geq 11\%$ and < 50% and diffuse i.e. $\geq 50\%$.

Positivity cut off was established with staining intensity of 2 or 3 combined with focal or diffuse pattern. Weak intensity and/or sporadic staining were regarded as negative [17].

Statistical evaluation

The SPSS software, version 21, was used for statistical analysis. Chi-square test and Z test for proportion were used to find relation between variables, *p* values of <0.05 were considered statistically significant.

Results

Studies showed that ketones promote mitochondrial “health” in cancer cells, effectively shutting off their apoptotic machinery, causes continuous proliferation. As such, ketone body utilization could have important implications for both cancer prevention, as well as the effective control of advanced metastatic disease. But whether ketone bodies production is a function of the cancer epithelial cells themselves or the cancer associated fibroblasts still need to be illustrated.

A total of 40 cases of invasive ductal carcinoma (NOS) were studied with a mean age of 47.83 years \pm 12.04. ACAT1 positive expression was seen in 87.5% cases of the cancer epithelial cells, while 67.5% cases were positive in CAFs, suggesting that ketone bodies consumption by the cancer epithelial cells is more efficient

than by CAFs. Kidney tissue was used as positive control showing cytoplasmic stain of renal tubules, (Figure 1 a, b and c).

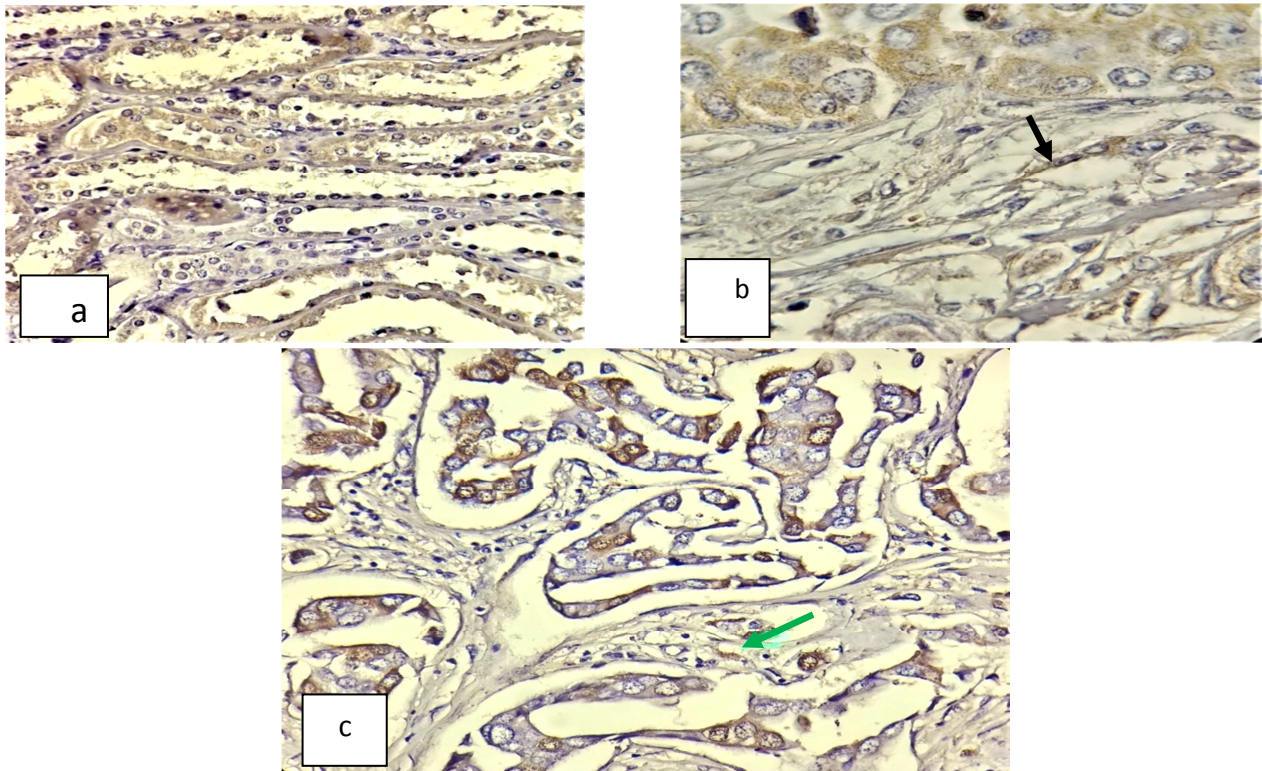


Figure-1 ACAT1 expression in control tissue and cancer.

(a) Kidney tissue used as positive control to show cytoplasmic staining in renal tubules (X400), (b) Staining intensity is intermediate in cancer epithelial cells and high in CAFs (arrow, oil X1000) (c) staining intensity is high in cancer epithelial cells and intermediate in CAFs (arrow) (X400)

HMGCS2 expression was positive in 97.5% of cases in the cancer epithelial cells and in 95 % of cases in CAFs, suggesting that both cancer compartments are highly efficient in production of ketone bodies. Liver hepatocytes were used as positive control and showed cytoplasmic staining, (Figure 2 a, b and c).

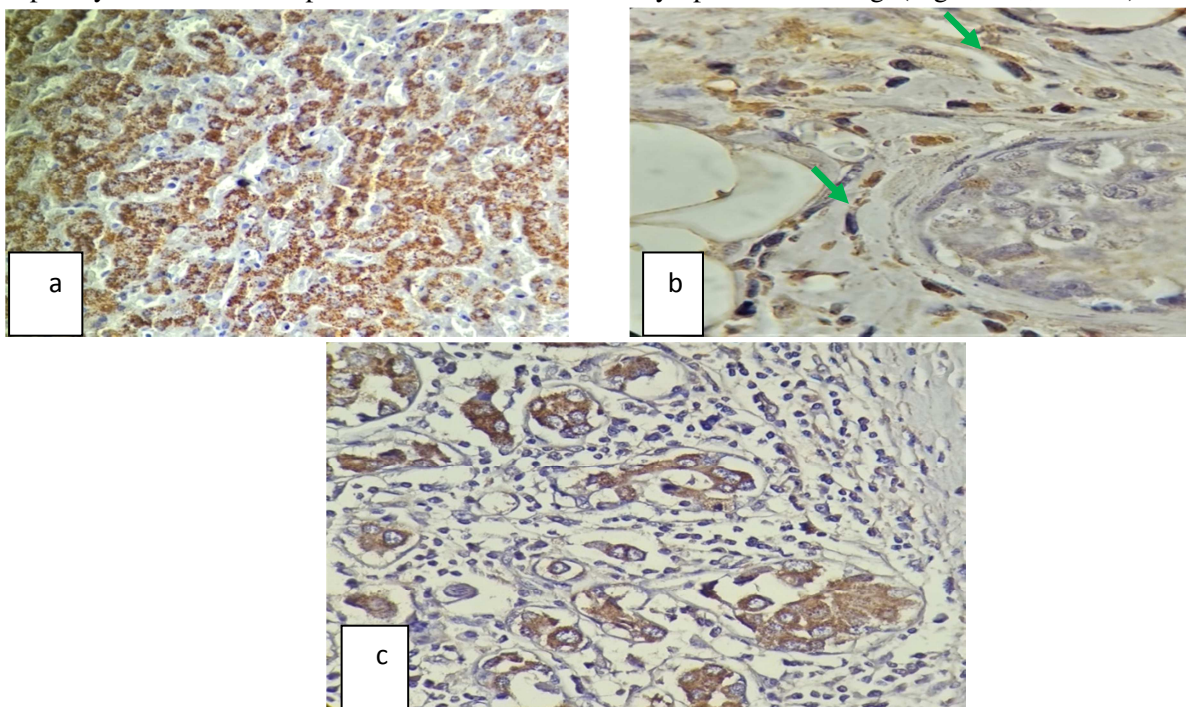


Figure-2 HMGCS2 in control tissue and cancer

(a) Hepatocytes as positive control showing high intensity cytoplasmic staining X400. (b) Staining intensity is high in CAFs (arrows) and low in cancer epithelial cells (oil X1000) (c) staining intensity is high of cancer epithelial cells and low in CAFs (X400).

Correlations of ACAT1 and HMGCS2 in cancer epithelial cells and cancer associated fibroblasts

Studying a single compartment of the tumor named the cancer epithelial cells (as Warburg did) and investigating its ability to either produce or utilize the ketone bodies; a correlation was done between the two antibodies. Table 1 shows that the cancer epithelial cells compartment express both enzymes in 87.5% of cases Therefore, these data indicate that in the majority of cases the cancer epithelial cells can produce and utilize the ketone bodies with no statistically significant difference between both processes (P.= 0.09).

Table 1 Correlation of HMGCS2 and ACAT1 positivity in cancer epithelial cells.

		HMGCS2 in cancer epithelial cells		P- value
		Positive No. (%)	Negative No. (%)	
ACAT1 in cancer epithelial cells	Positive No. (%)	35 (87.5%)	0 (0%)	0.09
	Negative No. (%)	4 (10%)	1 (2.5%)	

The idea of reverse Warburg effect was the energy production by stromal cell and utilization by cancer cells through the production of lactic acid. In this research an exactly comparable concept was investigated but through the production of ketone bodies instead of lactic acid and shows no statistical significant difference between the two compartments (P. = 0.23). From Table 2, we can estimate that in 95% of the cases the CAFs express HMGCS2 while cancer epithelial cells in cases 87.5% express ACAT1, suggesting that the majority of CAFs can produce ketone bodies and the majority of cancer epithelial cells can utilize.

Table 2 Correlation of HMGCS2 positivity in CAFs and ACAT1 in cancer epithelial cells.

		ACAT1 in cancer epithelial cells		P- value
		Positive No.(%)	Negative No.(%)	
HMGCS2 in CAFs	Positive No. (%)	34 (85%)	4 (10%)	0.23
	Negative No. (%)	1 (2.5%)	1 (2.5%)	

The difference significance was studied and showed a P. = 0.03 between the two compartments in their utilization of ketone bodies. Table 3 shows that 87.5% of cancer epithelial cells can utilize the ketone bodies as a source of energy compared to 67.5% of CAFs, suggesting that both compartments consume the ketone bodies but more efficiently by cancer epithelial cells.

Table 3 Correlation of ACAT1 positivity in both cancer epithelial cells and CAFs.

		ACAT1 in CAFs		P- value
		Positive No. (%)	Negative No. (%)	
ACAT1 in cancer epithelial cells	Positive No. (%)	26 (65%)	9 (22.5%)	0.03
	Negative No. (%)	1 (2.5%)	4 (10%)	

Expression of HMGCS2 was estimated and found in 97.5% of cases in cancer epithelial cells compared to 95% of cases in CAFs and statistically there was no significant difference between the two (P. = 0.55), suggesting that in both compartments the majority of cells can produce ketone bodies, (Table 4).

Table 4 Correlation of HMGCS2 positivity in both cancer epithelial cells and CAFs.

		HMGCS2 in CAFs		P- value
		Positive No. (%)	Negative No. (%)	
HMGCS2 in cancer epithelial cells	Positive No. (%)	38 (95%)	1 (2.5%)	0.55
	Negative No. (%)	0 (0%)	1 (2.5%)	

Discussion

This research highly supports the Warburg hypothesis and the concept of single compartment in feeding themselves, here in this research the cancer epithelial cells can produce and utilize efficiently the ketone bodies through expression of both mitochondrial enzymes in 97.5% and 87.5% respectively. In other words, the cancer epithelial cells have self dependence to fuel themselves.

ACAT1 positivity in cancer epithelial cells was demonstrated by Martinez-Outschoorn et al. (2012) who used Western blot analysis with isoform-specific antibodies and immunohistochemically stained paraffin embedded breast cancer tissue to confirm the overexpression of ACAT1 antibody in cancer epithelial cells [16,17].

Most of previous studies concentrate on the idea that the cancer two compartments have reciprocal relationship so as the cancer associated fibroblasts produce the energy needed for cancer epithelial cells growth and supporting the reverse Warburg effect. Sotgia, F et al. (2011,2012) [5,18] suggest that stromal cells that lack cav-1 will undergo aerobic glycolysis and feed cancer cells and if co-cultured with cancer cells promotes Cav-1 down-regulation in adjacent fibroblasts which enhance aerobic glycolysis, but when cultured under homotypic conditions, cancer cells have a very low mitochondrial mass this support the conventional Warburg effect. In other words, cancer epithelial cells can feed themselves but much less efficiently than if cultured with fibroblasts, which is highly consistent with this research in which both Warburg and reverse Warburg effects do occur in cancer [5, 18].

Our results show that both compartments in the tumor are capable of utilization of ketone bodies because both show positivity to ACAT1 enzyme as 87.5% and 67.5% in cancer epithelial cells and cancer associated fibroblasts respectively, i.e. when cancer epithelial cells utilize ketone bodies the tumor associated fibroblasts also can utilize them, which was up to our knowledge not fully clarified by other authors. ACAT1 enzyme in human fibroblasts is documented by Li et al. (1999) who used human fibroblast in location of human ACAT1 coding region, this supports that fibroblasts can utilize ketone bodies [19]. Our finding is consistent with Martinez-Outschoorn. et al. (2011) who Showed that fibroblasts and cancer cells shared parallel glucose uptake and they suggest that tumor cells and stromal fibroblasts in co-culture were metabolically coupled [20].

This study has shown that the cancer epithelial cells and cancer associated fibroblasts show cytoplasm positivity for HMGCS2 in 97.5% and 95% respectively, which means that both compartments have the enzyme needed for ketone body synthesis, this is consistent with Martinez-Outschoorn et al. (2012) who showed positivity in both compartments by using IHC stained paraffin embedded human breast cancer tissue and Western blot technique, but they did not clarify the epithelial positivity [16].

Bonuccelli, Gloria et al. (2010) also has demonstrated the expressing enzymes that need for ketone body production in fibroblast associated tumor microenvironment by using xenograft model for explanation of the role of 3-hydroxy-butyrate in increasing tumor size without significant increase in angiogenesis and they used a list of genes that are transcriptionally upregulated in human breast cancer epithelial cells, relative to the adjacent stromal cells [21].

Conclusions

In this research it is evident that each compartments of breast cancer can produce and utilize the ketone bodies. Through this research we can assume that ketogenic diet has no role in controlling breast cancer

growth since both compartments can efficiently produce and consume the ketone bodies i.e. fuel themselves in addition to the undeniable interaction between both. This may have some therapeutic impact on management of breast cancer.

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