Role of Angiotensin-Converting Enzyme and Vitamin D Receptor Gene Polymorphisms in Cancer Anorexia-Cachexia Syndrome

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ABSTRACT

The ubiquitin-proteasome pathway is a crucial connection between aberrant immune system activation, systemic inflammation and Cancer Anorexia-Cachexia Syndrome (CACS), a syndrome that culminates in hyper-activation of the ubiquitin-proteasome pathway. Angiotensin directly up-regulates this pathway, while vitamin D down-regulates it indirectly through the insulin-like growth factor-1 pathway. We investigated the genetic predisposition towards CACS in a cancer population, examining Insertion/Deletion (I/D) polymorphism of angiotensin-converting enzyme gene and *FokI* and *BsmI* polymorphisms of vitamin D receptor gene. Sixty-two cancer patients were recruited and divided into three groups: primary cachectic (C1, $n = 14$; dysmetabolic body weight loss $\geq$5% in 6 months); secondary cachectic (C2, $n = 34$; similar weight loss, mechanic or iatrogenic origin); and non-cachectic (NC, $n = 16$). C2+NC were merged in the control group. The three groups showed significant differences in average prognostic inflammatory nutritional index (C1: 26.4±23.4; C2: 5.4±5.6; NC: 0.37±0.5), C-reactive protein serum levels (C1: 6.6±2.1; C2: 2.4±2.2; NC: 1.0±2.0 mg/dl), albumin serum levels (C1: 3.1±0.6; C2: 3.5±0.4; NC 3.7±0.6 g/dl), weight loss (C1: 22±8; C2: 15±6.7; NC 5±6%) and life expectancy (C1: 6.4±3.3; C2: 25±28; NC: 45±25 months). However, none of the chosen polymorphisms showed any statistically significant correlation with CACS. The complexity of the changes of the immune system in the chronic inflammation state associated with CACS is far greater than expected and further studies are required to identify genetic independent markers of progression toward CACS.

Keywords: Weight Loss, Prognosis, Inflammation

1. INTRODUCTION

Cancer Anorexia-Cachexia Syndrome (CACS) is a dramatic and debilitating occurrence that can be observed at any stage of malignancy and has been described as a clinical syndrome accompanying cancer since ancient times (Bennani-Baiti and Walsh, 2009). We recently demonstrated that cancer patients with CACS have higher C-Reactive Protein (CRP) levels compared with non-cachectic cancer patients (Punzi *et al.*, 2012), thus reinforcing the hypothesis that the chronic inflammatory state commonly observed in advanced cancers is linked to the aetiology of CACS (MacDonald, 2011). Aberrant immune system activity

is a crucial aspect of the chronic systemic inflammation associated with CACS and it triggers a vicious cycle leading to further tumour growth and metastases. Although the interconnections between aberrant immune system activity and CACS are multifaceted, two specific pathways appear to be prominent; the ubiquitin-proteasome pathway (Camps *et al.*, 2006) and the vitamin D axis that includes vitamin D, the polymorphic vitamin D receptor (VDR), the vitamin D-binding protein (or Gc-protein) and its derivative, Gc protein-derived Macrophage Activating Factor (GcMAF), one of the most potent stimuli of the immune system (Strohle *et al.*, 2010; Ruggiero and Pacini, 2011).
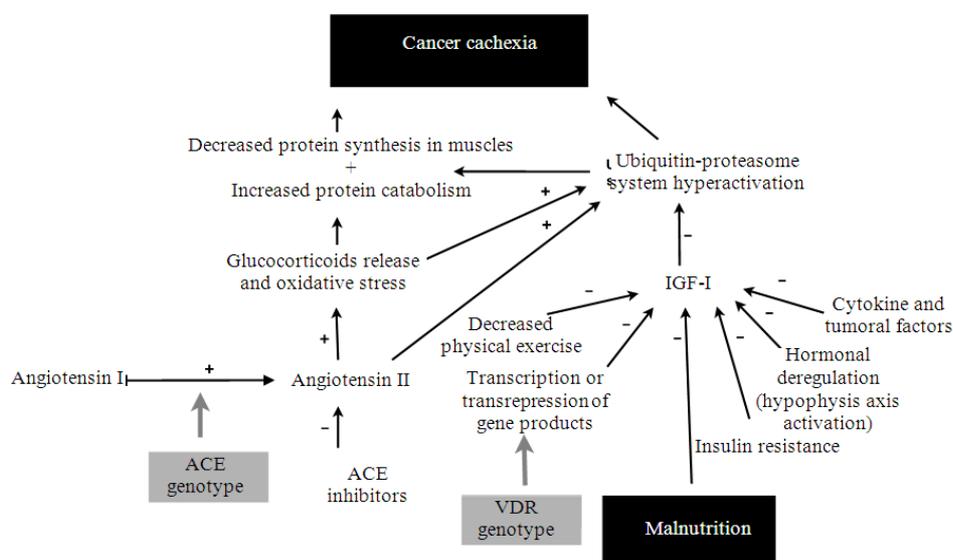
Fig. 1. Angiotensin-Converting Enzyme (ACE) and Vitamin D Receptor (VDR) genotypes in cancer anorexia-cachexia syndrome

As schematically illustrated in **Fig. 1**, the association between the ubiquitin-proteasome pathway, Angiotensin Converting Enzyme (ACE) and VDR polymorphic genotypes, CACS and the immune system is a complex one. In this regard, at the 2007 annual meeting of the American Society of Clinical Oncology, Vigano *et al.* (2007) presented a challenging work entitled "Angiotensin-Converting Enzyme Gene Polymorphism (ACEGP) and Cancer Cachexia (CC): Is there a link?" (Vigano *et al.*, 2007). In fact, it had been previously demonstrated that angiotensin I (ATI)-induced protein degradation was inhibited by the ACE inhibitor imidapril and/or the proteasome inhibitors lactacystin or MG132 (Sanders *et al.*, 2005). In Addition, it was demonstrated that angiotensin II (ATII) suppressed hepatic expression of Insulin-Like Growth Factor-1 (IGF-1) (Delafontaine and Akao, 2006) and increased cortisol serum levels (Ortiz *et al.*, 2010). These two events act synergistically on muscle protein breakdown with consequent liberation of free amino acids via the ubiquitin-proteasome pathway. Therefore, considering the pro-catabolic effects of ATII on skeletal muscle, mainly via the ubiquitin-proteasome pathway, our hypothesis was that ACE gene polymorphisms could be associated with different prognosis in CACS patients.

If ACE gene polymorphisms may be associated with a pro-catabolic effect, VDR gene polymorphisms on their part could be associated with an anti-catabolic effect. In fact, activated VDR acts as a transcriptional factor forming a heterodimer with the retinoid-X receptor and then binding to response elements on DNA, resulting in expression or repression of specific target genes (Moore *et al.*, 2006). Among these, the IGF-1 transcriptional pathway falls under the target of VDR modulation (Jurutka *et al.*, 2001). IGF-1, a potent anabolic signal, directly inhibits the ubiquitin-proteasome system, thus, at least hypothetically, counteracting the effects of ACE product (Stitt *et al.*, 2004). The ubiquitin-proteasome pathway appears thus to be located at a critical control point with ACE and VDR gene polymorphic genotypes possibly associated with opposite effects on the anabolic/catabolic balance.

Therefore, in this study we determined Insertion/Deletion (I/D) ACE gene polymorphism and *FokI* and *BsmI* VDR gene polymorphisms in cancer patients in order to establish whether specific polymorphic genotypes could be associated with the prognosis.

2. MATERIALS AND METHODS

After giving their informed consent, sixty-two cancer patients were randomly recruited in the Nutritional Unit of the Misericordia e dolce hospital in Prato, Italy. Age, gender, stage, primary cancer localization and ongoing therapy were randomly chosen. We divided the enrolled subjects into the following three groups: primary Cachectic (C1), secondary Cachectic (C2) and non-Cachectic (NC). The ranking criteria followed the guidelines set by the Italian Association of Medical Oncology (AIOM) in 2007 and are summarized in **Table 1** (AIOM, 2008). Accordingly, we had 14 patients in the C1 group, 32 in the C2 group and 16 in the NC group.

Table 1. Criteria for diagnosis of primary and secondary CACS, AIOM 2007

Primary CACS
Loss ≥ 5% of pre-illness weight within the last 6 months plus
Decreased muscle strength
Fatigue
Anorexia
Low lean mass index
Biochemichal alterations:
Increase in cytokine levels
Hb<12 mg/dl
Albumin < 3.2 mg kg^{-1}
Additional criteria for diagnosis of secondary CACS
CACS *plus* clinical evidence of
Gastrointestinal obstruction
Chemotherapeutic treatment, radiation therapy
Post-interventional malabsorption sindrome

CACS, cancer anorexia-cachexia syndrome; AIOM, Associazione Italiana di Oncologia Medica;
Hb, haemoglobin

As reported in **Table 1**, the diagnosis of cachexia followed the documented loss of 5% or more of body weight in the 6 months preceding the diagnosis of cancer. Despite the broad lack of consensus for this quantification, with many Authors assuming occurrence of CACS only after 10% body weight loss in 6 months, we postulated that in an actual clinical frame an early threshold was preferable. Secondary CACS depicted the frequent situation of a wasting syndrome derived from precise detectable causes. The two main tools to distinguish C1 from C2 were, therefore, targeted anamnesis and endoscopy/imaging. The unknowing patient, who involuntarily was losing weight with a conserved appetite or with a first manifestation of anorexia, was the prototype patient fitting the C1 criteria. The dyspeptic, dysphagic patient, who was losing weight after chemotherapy, surgery, radiation therapy, or because of severe stenosis at the level of the gastrointestinal tract, was a fitting model for C2. NC patients sometimes also lost weight. In this case, however, it was generally a therapeutic weight loss aiming to prevent complications in poly-morbid conditions, like heart failure, diabetes, respiratory distress.

We analyzed the three groups as separate entities during the epidemiological study, but we reduced the last two to a single one, the non-primary CACS group (NC = C2+NC), for the study of polymorphic genotypes, considering this as the control group with no presumable genetic predisposition towards weight loss. Each enrolled patient underwent accurate clinical examination, blood collection and DNA sampling. The clinical data of interest included age, tumor type, stage, pre-illness weight and timing of weight loss. Weight measurement in the ambulatory unit concluded the routine. Venous blood samples were taken and serum levels of CRP, pre-albumin, albumin and alpha-1-acid-protein were determined in the clinical laboratories of the hospital. We then calculated the Prognostic Inflammatory and Nutritional Index (PINI) score for each patient. Regrettably, approximately one of two patients taking part in the study deceased during the 2 year observation period. A retrospective analysis of life expectancy could therefore be traced, starting from the first day of cancer diagnosis.

DNA sampling was done on finger prick samples of approximately 50 µl. DNA extraction and purification were performed by QIAamp® DNA Blood Mini Kit (QIAGEN S.p.a., Italy). Purified DNA was then examined for *FokI* and *BsmI* VDR gene polymorphism and I/D ACE gene polymorphism after polymerase chain reaction amplification (SuperMini Cycler®, Mj Research, Inc., USA) using specific primers and the AccuPrime Supermix Kit (Invitrogen, Carlsbad, CA, USA) (Punzi *et al.*, 2012).

Investigation of the ACE gene I/D polymorphism required no restriction enzymes; once amplified, the genetic sequence of interest had different lengths, depending on the presence (I) or absence (D) of the fragment 288 bp length. On the contrary, to determine the allelic characteristic of the VDR gene, each amplified DNA fragment was treated with appropriate restriction enzymes (Punzi *et al.*, 2012). The restriction products of *FokI* and *BsmI* were sorted as F and f and B and b respectively, where capital letters indicate an absence of restriction sites and lower-case letters indicate their presence.

3. RESULTS

As expected, the clinical threefold subdivision of patients produced radically different groups in terms of average weight loss, intensity of inflammatory reaction, nutritional status, PINI score and life expectancy. It is important to notice that differences in average age and stage proved to be non-significant; we consider this observation of clinical relevance since it reinforces the notion that CACS is a stochastic event. C1 patients had the highest systemic inflammation, lowest nutritional status and dramatically poorest prognosis (**Table 2**). As previously described by our research group (Punzi *et al.*, 2012), CRP proved to be one of the most reliable and economic blood markers for CACS that a standard clinical laboratory can study (Mahmoud and Rivera, 2002). A standard nutritional status assessment usually includes the determination of albumin serum level, despite the open discussion regarding which albumin threshold should be chosen for the diagnosis of malnutrition.

Table 2. Patients' data

	C1		C2		NC	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	N
Age (years)	66.9±8.66	14	66.8±11.6	32	59.9±10.6	16
Stage	3.6±0.6	14	3.6±0.6	32	3.4±0.9	16
Gender	43% F	14	37% F	32	50% F	16
%Weight loss in 6 months	21.0±7.9	14	15.4±6.7	32	5.1±6.2	16
CRP (mg/dl)	6.6±6.3	9	2.4±2.2	29	1.0±2.0	16
Albumin (mg/dl)	3.1±0.6	10	3.5±0.4	29	3.7±0.6	15
PINI score	28	9	4	20	0.98	11
Average survival time after diagnosis of cancer (months)	6.4±3.3	9	26.1±27.6	12	45.3±25.0	7

C1, high risk; C2, intermediate risk; C3, normal. F, female

Table 3. Genotype frequencies in primary CACS and control groups

VDR gene polimorphysms	Genotype	C2 + NC (n = 48)	C1 (n = 14)	p
FokI	FF	0.28	0.36	0.81
	Ff	0.64	0.57	
	ff	0.08	0.07	
BsmI	BB	0.10	0.07	0,87
	Bb	0.53	0.50	
	bb	0.37	0.43	
ACE gene polymorphisms	DD	0.45	0.50	0,62
	ID	0.38	0.43	
	II	0.17	0.07	

C1, primary cancer anorexia-cachexia syndrome (CACS); C2 + NC, no presumable genetic disposition towards CACS; VDR, vitamin D receptor; ACE, angiotensin-converting enzyme

Table 4. Allele frequencies in primary CACS and control groups

VDR polymorphisms	Alleles	C2 + NC (n = 48)	C1 (n 14)	p
FokI	F	0.60	0.68	0.64
	f	0.40	0.32	
BsmI	B	0.36	0.32	0.63
	b	0.64	0.68	
ACE polymorphisms	D	0.64	0.71	0.41
	I	0.36	0.29	

C1, primary cancer anorexia-cachexia syndrome (CACS); C2 + NC, no presumable genetic disposition towards CACS; VDR, vitamin D receptor; ACE, angiotensin-converting enzyme

We set the cutoff for malnutrition at 3.5 mg/dl albumin (Harrison, 1998) and at 3.2 mg/dl for a diagnosis of severe malnutrition. The critics for albumin as a nutritional marker mainly revolve around two observations, namely that hepatic protein synthesis reprioritization in the acute inflammatory response is *per se* sufficient to precipitate albumin serum levels, despite normal nutritional status and that compensatory mechanisms keep albumin levels high during prolonged insufficient protein intake through depletion of skeletal muscle tissue. Although high CRP levels are good indexes for systemic inflammation, poor levels of albumin cannot distinguish starvation from chronic systemic inflammation. Therefore, in order to draw a comprehensive picture of the three patient groups, we combined the collected blood values into a single score, the PINI score a reliable tool capable of estimating the risk of complications during hospitalization of chronically ill patients (Yates, 1980). PINI is calculated by dividing the product of serum alpha-1-glycoprotein and CRP levels by that of albumin and pre-albumin.

Prognostic Inflammatory and Nutritional Index:

$$\frac{alpha-1\ acid-glycoprotein\ (mg/dl)\ x\ CRP\ (mg/dl)}{albumin\ (g/dl)\ x\ prealbumin\ (mg/dl)}$$

Where:

PINI > 30 = predicts a very high risk of complications during hospitalization [21-30] predicts a high risk of complications during hospitalization

[11-20] = Predicts an intermediate risk

[1-10] = Predicts a low risk

< 1 = Normal

As reported in **Table 2**, the C1 group fell in the high-risk category, C2 in the intermediate and NC in the normal. The survival rate of the three groups confirmed the nutritional prognosis.

Given the significant differences of the three populations, in order to investigate the genetic predisposition towards CACS, we isolated the C1 group (*n* = 14) and created a reference group merging C2 and NC pools (*n* = 48), which presumably had no genetic

predisposition towards CACS. The study of genotypic frequency results from the proportion between the number of subjects harboring a specific genotype and the total number of subjects. Below is the formula for the frequency of an exemplificative FF genotype; genotype frequencies in primary CACS and control groups are shown in **Table 3**:

$$FF\ genotype\ =\ number\ of\ FF\ subjects$$
$$/total\ examined\ subjects\ (FF\ +\ Ff\ +\ ff)$$

Comparing the genotype frequencies of the VDR gene between the group of primary cachectic patients and the control group, no significant difference could be found (p>0.05). Comparing the genotype frequencies of the ACE gene between the same pools of patients, the same result was obtained. The distribution of allele frequencies was then calculated both for the VDR and ACE gene polymorphisms on the basis of genotype frequencies (**Table 4**). Below is the formula for calculating allele frequency:

$$F\ allele\ frequency = genotype$$
$$FF\ frequency + \tfrac{1}{2}\ Ff\ genotype\ frequency$$
$$f\ allele\ frequency = 1 - F\ allele\ frequency$$

Allele distribution for both ACE and VDR genes showed no significant difference between the primary cachectic group and the control group, for any of the studied polymorphisms (**Table 4**).

4. DISCUSSION

Genetics is a promising field for the study of predisposition towards CACS, because CACS itself is an apparently stochastic event, occurring at random among individuals who are not significantly different in terms of age, stage and tumor type as confirmed in this study. Nevertheless, according to the results of our observations, the answer to the question "Angiotensin-Converting Enzyme Gene Polymorphism (ACEGP) and Cancer Cachexia (CC): Is there a link?" appears to be negative. In fact, both allele frequencies and genotypic frequencies of the ACE gene polymorphisms were not significantly associated with clinical progression of CACS. A similar conclusion can be drawn for VDR gene polymorphisms. These conclusions notwithstanding, we cannot rule out the possibility that different results could be obtained by increasing the size of the sample to be examined. In addition, although the random selection of

patients was a precise choice of this study reflecting the clinical actuality of most hospitals, we cannot rule out the possibility that in certain sub-groups of CACS patients the results might be different.

5. CONCLUSION

In conclusion, our study demonstrates that the complexity of the changes of the immune system in the chronic inflammation state associated with CACS (Khatami, 2011) is far greater than expected and further studies are required to identify genetic independent markers of progression toward CACS that, as of today, remains a stochastic, unpredictable event.

6. ACKNOWLEDGEMENT

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