Serum amyloid A1 polymorphisms as risk factors in oesophageal squamous cell carcinoma

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KEY MESSAGES

1. Serum amyloid A1 provides 100% sensitivity and 100% specificity for early detection of oesophageal squamous cell carcinoma. The choice of using serum or plasma samples is critical for circulating serum amyloid A detection.
2. The median survival time of patients with serum amyloid A1.3/1.5 genotype was shorter than that of patients with other genotypes (10.63 months vs 20.41 months, P=0.004). This was likely due to the extra high-circulating interleukin 6 concentrations.

Hong Kong Med J 2019;25(Suppl 7):S4-8
HMRF project number: 01120886

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Introduction

Oesophageal carcinoma is highly metastatic and often fatal. Most such patients do not survive for >1 year after diagnosis, and the 5-year survival rate is <10%. Early detection of oesophageal squamous cell carcinoma (OSCC) is difficult because the symptoms are not obvious until the tumour is advanced and metastatic at the time of presentation. It is essential to identify an early biomarker for this disease.

Serum amyloid A is an acute-phase high-density lipoprotein–associated apolipoprotein, which is dramatically elevated (up to 1000-fold) in serum following injury, inflammation, and cancer. SAA has been used as a cancer biomarker in many tumour types including OSCC.[1] Elevated SAA level is associated with cancer progression and survival. SAA is a generic term for a family of acute-phase proteins encoded by various *SAA* genes with a high genetic variation.

During the acute-phase response, the hepatic biosynthesis of SAA is up-regulated by pro-inflammatory cytokines including interleukin (IL)-1, IL-6, and tumour-necrosis factor.[2] There is SAA induction of different extracellular matrix degradation enzymes such as matrix metallopeptidases (MMPs).[3] SAA plays a role in the modulation of inflammatory and immune responses via inducing MMP-9 in human monocytic cells.[4] Thus, using SAA, IL-6, IL-8, and MMP-9 as serum biomarkers may increase sensitivity and specificity for OSCC detection. We therefore compared their usefulness in OSCC diagnosis and prognosis with a classic tumour biomarker – squamous cell cancer antigen (SCCA). Both plasma and serum samples of OSCC patients were included to examine the protein levels of interest. In addition, we investigated whether particular *SAA1* genotypes are associated with the elevated SAA, IL-6, IL-8, and MMP-9 levels in OSCC patients. In addition, gene expression in OSCC tumour tissues was also assessed. We aimed to determine (1) whether *SAA1* polymorphisms are correlated with the *SAA1* and its related gene expression in the OSCC tumour tissues, and (2) whether *SAA1* polymorphisms have any functional role in OSCC development.

Methods

This study protocol was approved by the Institutional Review Board of The University of Hong Kong / Hospital Authority Hong Kong West Cluster (Ref No. UW 13-393), and written consent was obtained from all patients. A total of 226 patients with OSCC who underwent oesophagectomy with no preoperative chemoradiotherapy between 2000 and 2012 at the Department of Surgery, Queen Mary Hospital were included. The genomic DNA from blood samples of these patients and of 295 healthy controls (supplied by the Hong Kong Red Cross) were collected for SAA1 genotyping.

ELISA was performed to detect circulating proteins in plasma and serum samples of 85 of the OSCC patients and in plasma samples of 100 of the healthy controls. For gene expression analysis, matched non-tumour biopsies and OSCC biopsies (from 60 of the 85 OSCC patients) were used. The gene expression of *SAA1*, *IL-6*, *IL-8*, *MMP-9* was determined by quantitative PCR using previously described methods.[5] SAA1-genotyped OSCC patients, healthy controls, and hospital controls (non-cancer patients in Queen Mary Hospital) were compared in terms of plasma/serum SAA (Abcam), IL-6 (R&D Systems), IL-8 (R&D Systems), MMP-9

(R&D Systems), and SCCA1 (Biomatik). The use of hospital controls was for adjusting confounding factors such as associated chronic inflammatory states. PCR amplification and DNA sequencing were carried out for blood samples of 226 OSCC patients and healthy controls as described.[5]

Associations between pathological variables of OSCC patients and gene and protein expression of *SAA1* were analysed using SigmaPlot (Systat Software) and SPSS (Version 19, IBM, Armonk [NY], USA). The student's *t* test was used unless stated otherwise. A P value of <0.05 was considered statistically significant.

Results

SAA1.1, *1.3*, and *1.5* were the major SAA1 isoforms observed in both OSCC patients and healthy people in Hong Kong. *SAA1.1/1.1*, *SAA1.3/1.3*, *SAA1.5/1.5*, *SAA1.1/1.3*, *SAA1.1/1.5*, and *SAA1.3/1.5* were the six major *SAA1* genotypes (Fig 1). Patients with *SAA1.3/1.5* genotype (n=45) had a shorter median survival time than those with other *SAA1* genotypes (10.63 months vs 20.41 months, P=0.004). The Kaplan-Meier survival analysis confirmed that *SAA1.3/1.5* genotype was associated with worse overall survival, and the longest survival time was observed in patients with the *SAA1.5/1.5* genotype (41.07 months). Thus, the *SAA1.3/1.5* variant was identified as a predictive biomarker of poor survival.

Pre-treatment OSCC patients (n=85) and healthy controls (n=100) were compared in terms of plasma levels of SAA, IL-6, IL-8, and MMP-9, as well as the conventional biomarker SCCA1 level. The plasma SAA level was ~10-fold more in OSCC patients than in healthy controls (536.86 ng/mL vs 50.06 ng/mL, $P=3.38 \times 10^{-59}$, Fig 2). In contrast, the serum levels of SAA and SCCA1 in the same patients were ~7-fold less than the plasma levels of SAA and SCCA1. Compared with hospital controls, OSCC patients only had a 1.43-fold increase in serum SAA (P=0.00083) and no significant elevation of serum levels of SCCA1, IL-6, IL-8, or MMP-9. Patients with high plasma and serum IL-6 levels showed poorer overall survival. High plasma/serum IL-6 levels in *SAA1.3/1.5* patients were likely to contribute to the poor survival of ~20% of OSCC patients (Fig 3).

The sensitivity and specificity of the circulating SAA, IL-6, IL-8, MMP-9, and SCCA1 for OSCC detection at different stages were calculated by

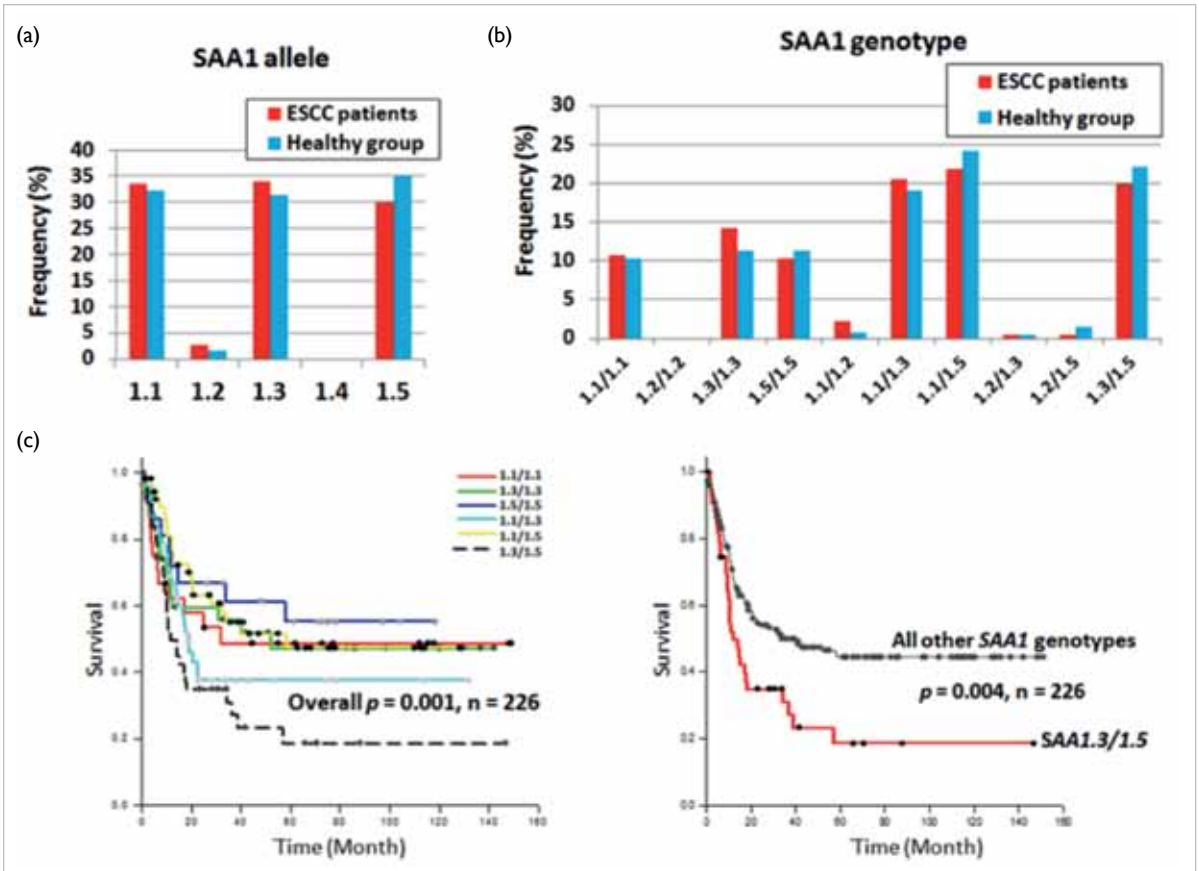


FIG 1. Frequency of (a) *SAA1* alleles and (b) *SAA1* genotypes in patients with oesophageal squamous cell carcinoma (OSCC). (c) The overall survival of patients with different major *SAA1* genotypes and the overall survival of patients with *SAA1.3/1.5* or other five major *SAA1* genotypes.

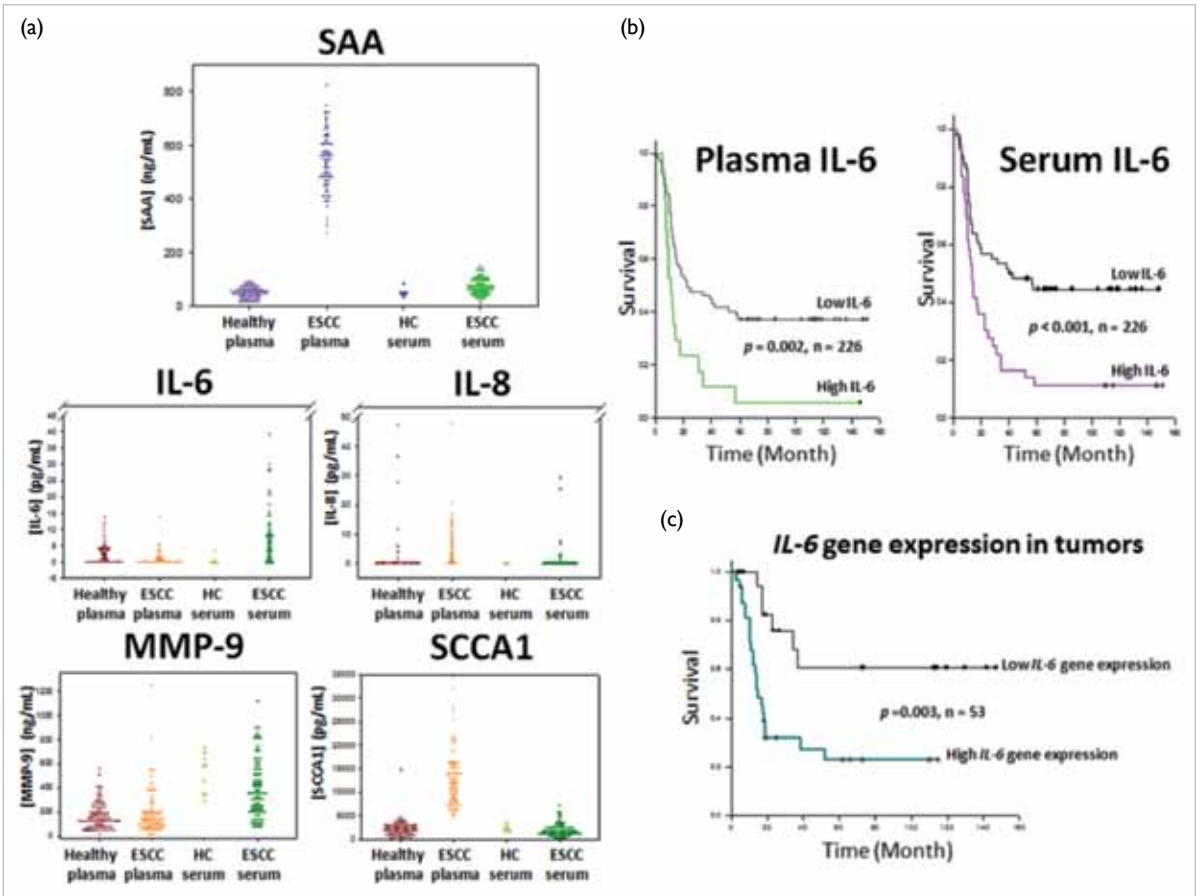
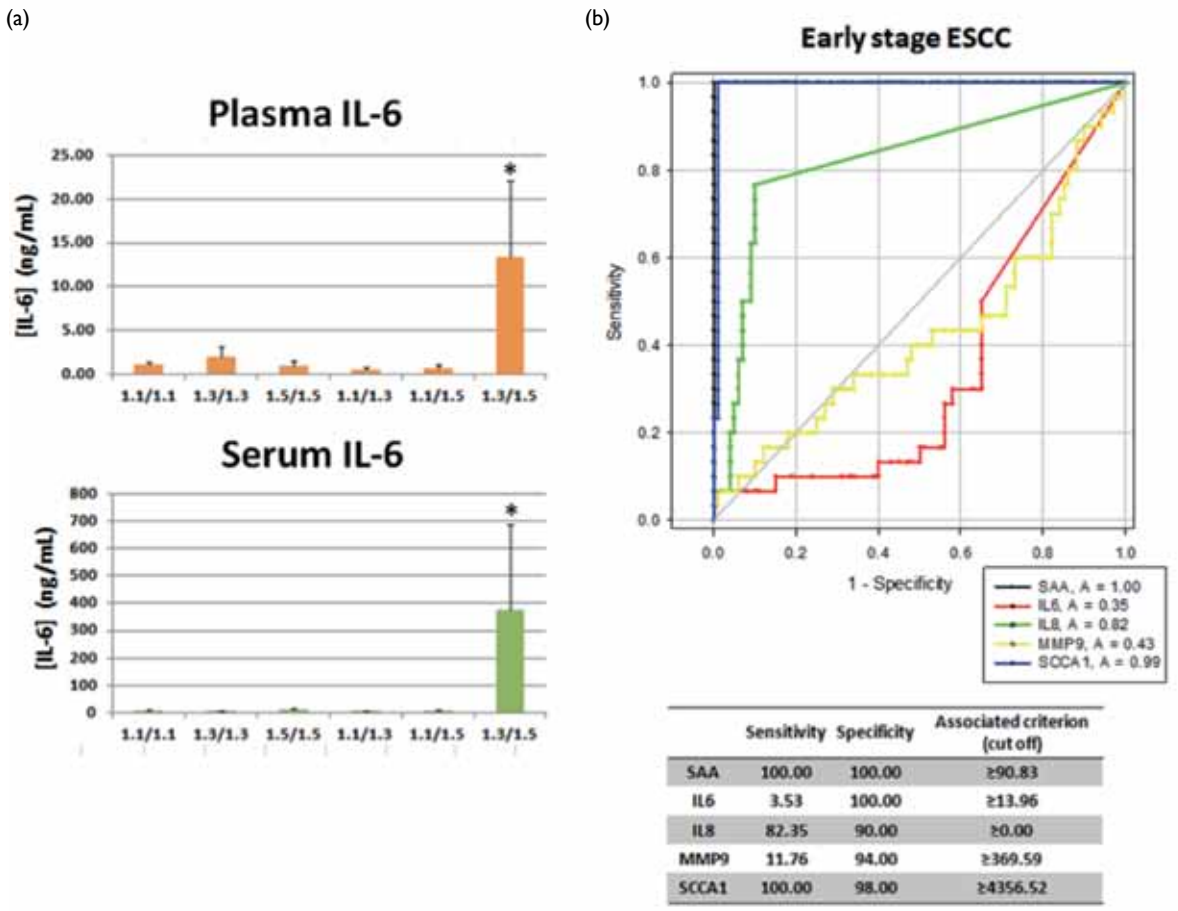


FIG 2. (a) Circulating SAA, IL-6, IL-8, MMP-9, and SCCA1 protein levels in plasma and sera of pre-treatment patients with oesophageal squamous cell carcinoma (OSCC), in plasma of healthy controls, and in sera of hospital controls. (b) Circulating SAA and IL-6 are prognostic markers. (c) Kaplan-Meier analysis showed that high *IL-6* gene expression is a marker for poor prognosis.

comparing the plasma levels of these proteins in OSCC patients and healthy controls. The plasma SAA and SCCA1 levels were elevated ~10-fold ($P=7.34 \times 10^{-20}$) and ~6-fold ($P=7.51 \times 10^{-10}$), respectively, in the early-stage (stages I and II) OSCC patients. Plasma SAA was better than plasma SCCA1 in detecting early-stage OSCC (specificity, 100% vs 99%; sensitivity, 100% vs 100%; area under the receiver operating characteristic curve, 1.00 vs 0.99; Fig 3). The circulating IL-6, IL-8, and MMP-9 had much lower sensitivity and specificity than either the plasma SAA or SCCA1. Thus, plasma SAA alone is an ideal biomarker for early OSCC detection.

Tumours with *SAA1.3/1.5* genotype showed the highest gene expression of *SAA1*, *IL-6*, and *MMP-9* and second highest *IL-8* expression among all major *SAA1* genotypes. The high expression levels of *SAA1* (P=0.011) and *MMP-9* (P=0.0097) was associated with *SAA1* genotype, whereas the high IL-6 gene expression was not associated with *SAA1.3/1.5* genotype (P=0.079). The Kaplan-Meier survival analysis showed that high levels of *IL-6* gene expression were associated with poorer survival (P=0.003). Circulating SAA level and gene expression of *SAA1* were not correlated (r=0.0062, P=0.972). Thus, the liver (rather than the tumour) was the major producer of the circulating SAA protein in OSCC patients.

To study the functional roles of these *SAA1* isoforms in OSCC development, *SAA1.1*, *1.3*, and *1.5* were ectopically expressed in three OSCC cell lines. Tumourigenicity was suppressed by restoration of *SAA1.1* and *SAA1.3*. This is in contrast to the appearance of tumours for the *SAA1.5*-expressing cells. The effects of the three *SAA1* isoforms in OSCC angiogenesis was assessed using the tube-forming ability of human umbilical vein endothelial cell tube formation assay. The *SAA1.5*-expressing cell line could not significantly reduce tube formation (P=0.283), whereas the *SAA1.1* and *SAA1.3* reduced by about 35.4% and 37.93%, respectively, of the human umbilical vein endothelial cell tube formation. The in vivo anti-angiogenic activities of the same *SAA1*-expressing cells were



	Sensitivity	Specificity	Associated criterion (cut off)
SAA	100.00	100.00	≥90.83
IL6	3.53	100.00	≥13.96
IL8	82.35	90.00	≥0.00
MMP9	11.76	94.00	≥369.59
SCCA1	100.00	98.00	≥4356.52

FIG 3. (a) Levels of plasma and serum IL-6 in the *SAA1*-genotyped samples. (b) Receiver operating characteristic curves of plasma levels of SAA, IL-6, IL-8, MMP-9, and SCCA1 in detection of early-stage oesophageal squamous cell carcinoma (OSCC).

assessed in mice with the matrigel plug assay. The numbers of microvessels in gel plugs of the *SAA1.1*- and *SAA1.3*-expressing cells reduced by 30.6% and 42.6%, respectively, compared with the vector-alone controls. The number of microvessels of the *SAA1.5*-expressing cells decreased by 8.15%, which was not significant.

Discussion

Plasma SAA is an ideal early detection marker for OSCC. The choice of using plasma or serum samples is critical for the detection of circulating SAA and SCCA1 proteins in cancer patients. Two possible reasons for varying protein levels in plasma and serum are: (1) SAA protein is more stable in plasma with a high protein matrix; and (2) SAA directly interacts with platelets to modulate platelet aggregation, and thus the blood coagulation can sequester most SAA protein in the clotted blood and result in reduced SAA protein in the serum samples. Results of the receiver operating characteristic curve analysis suggest that both plasma SAA and SCCA1 proteins are equally effective for detection of early OSCC. When hospital controls are included, plasma SAA has higher specificity and sensitivity than plasma SCCA1.

OSCC patients with *SAA1.3/1.5* genotype showed the shortest survival time (10.63 months). The abnormal high levels of circulating IL-6, IL-8, and MMP-9 in patients with *SAA1.3/1.5* genotype could be a reason for this short survival time. High-circulating IL-6 was able to stratify poor survival from good survival. Furthermore, the gene expression levels of *SAA1*, *IL-6*, and *MMP-9* were highest in patients with *SAA1.3/1.5* genotypes. Thus, poorer survival outcome was associated with OSCC patients with *SAA1.3/1.5* genotype.

Functional assays for the three *SAA1* variants (*SAA1.1, 1.3, 1.5*) in various OSCC cell lines showed that *SAA1.1* and *1.3* could suppress both tumour formation and angiogenesis, whereas *SAA1.5* had no significant effects. These results suggest that *SAA1.1* and *1.3* have the tumour suppressive function by inhibiting angiogenesis in the primary tumours. The *SAA1.5* seems to encode a defective SAA1 protein in

the anti-angiogenic function.

Acknowledgements

This study was supported by the Health and Medical Research Fund, Food and Health Bureau, Hong Kong SAR Government (#01120886). We thank the Hong Kong Red Cross for providing blood samples from healthy individuals. We also thank Dr Didier Trono for the supply of the lentiviral vectors, pWPI, pMD2.G, and psPAX2.

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