Increased plasma concentrations of anterior gradient 2 protein are positively associated with ovarian cancer

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ABSTRACT

Ovarian cancer is often asymptomatic and is diagnosed at an advanced stage with poor survival rates, thus there is an urgent need to develop biomarkers for earlier detection of ovarian cancer. In the present study, we demonstrate for the first time that the previously reported metastasis-inducing protein AGR2 (anterior gradient protein 2) can be detected in the blood of ovarian cancer patients. Using a newly developed ELISA, we show significantly increased concentrations of AGR2 protein in plasma from cancer patients relative to normal controls. Plasma AGR2 concentrations were highest in stages II and III ovarian cancer patients and were similarly elevated in patients with both serous and non-serous tumours. The identification of elevated plasma concentrations of AGR2 may provide a useful biomarker to aid in the discrimination of normal and ovarian cancer patients particularly when used in combination with CA125.

INTRODUCTION

Ovarian cancer is the most lethal of gynaecological cancers and represents one of the most frequent causes of cancer-related deaths in women in Europe and the United States. Since early stage ovarian cancer is often asymptomatic, the disease is usually diagnosed at an advanced stage, with 5-year survival rates of less than 30% arguing that improved early detection is paramount for enhanced survival [1]. At present, CA125 represents the best characterized and most widely used ovarian cancer biomarker, being elevated in more than 80% of patients with epithelial ovarian cancer, but only in approximately 50% of patients with stage I disease [1]. Therefore, there is an urgent need to develop novel diagnostic tests to be able to detect ovarian cancers as early as possible, when survival and treatment opportunities are greatest.

AGR2 (anterior gradient 2) protein is the human homologue of the cement-gland XAG-2 protein that was previously described in Xenopus laevis, where this protein has been shown to be a crucial factor involved in cellular differentiation and development [2]. In normal mammalian tissue, AGR2 is found in mucin-containing cells and may play a role in the production of intestinal MUC2 [3]. AGR2 mRNA has been shown to be co-expressed with ER (oestrogen receptor) in human breast cancer cell lines and in human breast cancer tissue [2,4]. AGR2 was also shown to be markedly elevated in the majority of prostate

Key words: anterior gradient protein 2 (AGR2), biomarker, CA125, ELISA, ovarian cancer.

Abbreviations: AGR2, anterior gradient protein 2; AUC, area under the curve; ESI, electrospray ionization; HRP, horseradish peroxidase; MAb, monoclonal antibody; MALDI, matrix-assisted laser-desorption ionization; PAb, polyclonal antibody; rhAGR2, recombinant human AGR2; MS/MS, tandem MS; ROC, receiver operator characteristic; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; TOF, time-of-flight.

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carcinomas compared with matched adjacent benign tissue, and immunohistochemical analysis showed that AGR2 expression was highly restricted to the secretory epithelial cells of the prostate gland [5].

Expression of an AGR2 cDNA in a benign non-metastatic rat mammary tumour cell line, Rama 37, led to highly significant lung metastasis of these tumour cells in a rat model, suggesting that the AGR2 gene product plays a pivotal role in inducing metastatic spread of tumour cells in vivo [4]. In addition, NIH3T3 cells stably transfected with AGR2 demonstrated enhanced foci formation and anchorage-independent growth in vitro and led to the establishment of tumours in nude mice [6], while silencing AGR2 in pancreatic tumour cells reduced cellular proliferation and invasion and improved sensitivity to the chemotherapy drug gemcitabine [7].

Part of the mechanism of action of AGR2 on cell growth and survival may be mediated by attenuating p53 activity, at least in oesophageal disease [8].

Limited results suggest that AGR2 is a differentially regulated gene in ovarian epithelial cancer. Gene profiling studies identified AGR2 as a significantly up-regulated gene in ovarian carcinoma, particularly in mucinous tumours [9]. A further study examining microdissected mucinous tumours of the ovary identified AGR2 as an up-regulated gene and showed by immunohistochemical staining that AGR2 was absent in normal ovarian epithelium, whereas mucinous cystadenomas and adenocarcinomas express AGR2 [10].

Taken together, the relatively low or absent expression of AGR2 in normal tissues and the up-regulated expression of AGR2 in several human cancers, including ovarian tumours, suggests that AGR2 may represent a molecular marker for tumour progression. Although the AGR2 gene contains a signal sequence suggestive of protein secretion and AGR2 appears to be secreted into the medium by some tumour cell lines [5,7], there is currently no evidence that AGR2 protein is secreted into the circulation in healthy humans or in human cancer patients. The aim of the present study was to determine whether plasma immunoreactive AGR2 could be detected and whether circulating levels of AGR2 were increased in ovarian cancer patients relative to controls. Using a newly developed ELISA test, we now demonstrate, for the first time, that AGR2 protein can be detected in the blood of ovarian cancer patients at a significantly higher concentration than in normal controls and may provide some benefit as a diagnostic marker when used in combination with CA125.

MATERIALS AND METHODS

Immunohistochemical staining of AGR2
A small set of archival paraffin-embedded specimens of ovarian tissue were obtained with informed consent from women presenting for surgery at the Royal Women’s Hospital to determine whether AGR2 could be localized in ovarian cancer specimens. Local Ethics Committee approval was obtained, and ovarian carcinoma classification and grading [based on WHO (World Health Organization) histological classification] were as reported as part of the clinical pathology diagnosis. Specimens tested comprised normal ovarian tissue and neoplastic lesions that ranged from benign cystadenomas to borderline tumours and carcinomas of varied differentiation and grade.

A previously described affinity-purified rabbit PAB (polyclonal antibody) raised against rhAGR2 (recombinant human AGR2) was used for immunohistochemical localization studies [4,11]. The specificity of this affinity-purified antibody for AGR2 has been rigorously tested in both Western blot and immunohistochemical studies [11]. Paraffin sections (4 μm) were cut, antigen retrieval was performed in 10 mM citrate buffer at pH 6.0 in a microwave oven for 10 min and endogenous peroxidase activity was blocked with 3 % H2O2/methanol. Sections were incubated with the anti-AGR2 antibody diluted 1:500 in TBS (Tris-buffered saline; 20 mM Tris/HCl, pH 7.4, and 150 mM NaCl) containing 3 % (w/v) skimmed milk powder for 2 h at room temperature (22 °C). The bound antibodies were detected using biotinylated goat anti-(rabbit IgG) in TBS containing 0.5 % BSA, followed by incubation with streptavidin–HRP (horseradish peroxidase) (Chemicon IHC Select secondary detection system; Millipore) and 3,3′-diaminobenzidine (Dako). Sections were counterstained with Mayer’s haematoxylin and mounted.

Control and ovarian cancer plasma samples
Samples used for measurement of AGR2 and CA125 comprised blood samples collected from healthy age-matched women and women at the time of diagnosis of ovarian cancer and prior to commencing treatment. The Mercy Hospital for Women Human Research and Ethics Committee approved the project. All case samples and part of the control sample set used in the present study were provided by the Biobank at Peter MacCallum Cancer Research Institute (East Melbourne, Victoria, Australia), and all subjects participated in the study after signing an informed written consent. Blood (10 ml) was collected via vena puncture into EDTA vacutainer tubes, and samples were centrifuged at 1000 g for 10 min within 20–30 min of collection. Plasma was stored as 250–1000 μl aliquots at −80 °C until assayed. The sample population comprised 61 controls (mean ± S.D. age = 50 ± 9) and 46 ovarian cancer patients (mean ± S.D. age = 60 ± 14) that included serous, mucinous, clear cell and endometrioid tumours of varying stages (stages I—III only) as outlined in Table 1. Ovarian tumour classification was based on the FIGO staging
Measurement of AGR2 and CA125

A 96-well plate sandwich ELISA was developed to quantify concentrations of AGR2. A MAb to human AGR2 used in the ELISA as capture antibody was affinity-purified from the patented hybridoma clone 7A10 (PCT/GB2007/003235), produced against a peptide (Eurogentec S.A.) unique to human AGR2 and not present in human AGR3 [12]. The specificity of this MAb has been previously tested by Western blot analysis and was shown to react with native AGR2 and rhAGR2, but not with the closely related AGR3 [12]. The second antibody used in the ELISA was a biotinylated affinity-purified rabbit anti-AGR2 PAb (polyclonal antibody) raised against full-length rhAGR2 as previously described [4]. Quantitative ELISA assays were established in Nunc-Immuno 96-well MaxiSorp plates after coating with MAb diluted to 62.5 ng/ml in 100 mM sodium carbonate, pH 9.6, at 4 °C for 18 h. The capture antibody was removed, and the plate was washed three times with wash buffer (50 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl and 0.05% Tween 20) prior to 60 min incubation with 200 μl of blocking buffer (Superblock; Pierce). Human plasma samples in duplicate and rhAGR2 calibrators for construction of the standard curve (100 μl) were added to the wells and incubated for 2 h at 37 °C. The wells were washed as above and 100 μl of the biotinylated PAb (1:200 dilution) was added, and incubation at 37 °C continued for 2 h. Following incubation with the detection antibody and three additional washes, neutravidin-conjugated HRP diluted 1:1000 in dilution buffer (Pierce) was added for 30 min, and a colour reaction was developed by the addition of 80 μl of one-step Ultra TMB substrate (Pierce) for 25 min. The reaction was stopped by addition of 80 μl of 2 M H2SO4, and the absorbance was read at 450 nm using a Multiskan Ascent plate reader (Thermo Scientific).

CA125 concentrations were quantified for comparison using the Roche CA125 Elecsys II assay kit on a Roche modular E170 platform.

Statistics and ROC (receiver operator characteristic) curve analysis

Multiple group comparisons of measured biomarker values were assessed by the Kruskal–Wallis test, and Dunn’s multiple comparison was used as post-hoc test to determine differences between groups. A P value of <0.05 was considered to be statistically significant.

A multivariate classification model was generated using a boosted logistic regression algorithm with Weka GUI Chooser software [13]. The generated algorithm reported a probability distribution value between 0 and 1.000 (i.e. the likelihood that each sample came from a woman with ovarian cancer, that is ρP) for each patient sample. A threshold value of 0.500 was used for classification of samples based on ρP, values of >0.500

Table 1  Distribution of ovarian tumour types and stages of ovarian cancer patients used for plasma AGR2 and CA125 measurements

Ovarian tumours were classified according to the FIGO staging system; no stage IV tumours were available for the present study.

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>All tumours</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Unstaged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous (n)</td>
<td>29</td>
<td>3</td>
<td>17</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Mucinous (n)</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Endometrioid (n)</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clear cell (n)</td>
<td>2</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Mixed Mullerian (n)</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untyped (n)</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (n)</td>
<td>46</td>
<td>9</td>
<td>26</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2  Immunohistochemical staining of ovarian tissue for AGR2 by cellular differentiation

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Positive AGR2 staining/total number of specimens (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface epithelium</td>
<td>0/5</td>
</tr>
<tr>
<td>Cystadenomata</td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>2/3</td>
</tr>
<tr>
<td>Mucinous</td>
<td>2/2</td>
</tr>
<tr>
<td>Borderline tumours</td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>5/5</td>
</tr>
<tr>
<td>Carcinomas</td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>7/10</td>
</tr>
<tr>
<td>Endo</td>
<td>3/3</td>
</tr>
<tr>
<td>CC</td>
<td>1/1</td>
</tr>
</tbody>
</table>

being classified as ovarian disease and samples with a calculated value < 0.500 being classified as normal. The probability values were used to generate ROC curves for comparison of the AUC (area under curve).

RESULTS

Immunohistochemical staining of AGR2 in ovarian tissue

Preliminary investigation of expression of AGR2 in benign and neoplastic ovarian epithelium was performed by standard immunohistochemistry using a specific anti-AGR2 PAb and a small number of ovarian tissue sections (Table 2). The neoplastic lesions ranged from benign cystadenomas to borderline tumours and carcinomas of varied differentiation and grade. No expression was detected in the non-neoplastic ovarian surface epithelium, whereas the majority of tumours expressed AGR2 within the neoplastic epithelium (Figure 1b and Table 2).

Isolation and identification of circulating AGR2 protein in plasma

The trypsin digest of immune-purified material derived from pooled ovarian cancer patient plasma was analysed by MALDI–TOF/TOF MS. AGR2 protein was identified by matching of five peptides with a total coverage of 23 % of the mature protein sequence that excludes the signal sequence that is predicted to be cleaved prior to secretion (Figure 2). One peptide, RDTTVKPGAKK, is unique to AGR2 and is not present in the closely related AGR3 (UniProt accession no. Q8TD06). Unequivocal confirmation of two peak identities was obtained by LC–ESI (electrospray ionization)–MS/MS(tandem MS)-based sequence analysis. These results confirm the identity of the immunoreactive species bound by the MAb as AGR2 and show for the first time that AGR2 protein has been detected and identified in the plasma of cancer patients.

Plasma concentrations of AGR2 and CA125 in ovarian cancer patients or healthy controls

Using a newly developed sandwich ELISA, AGR2 immunoreactivity was measured in human plasma from control individuals and ovarian cancer patients. As predicted by the specificity of the capture MAb used, the present ELISA assay showed no cross-reactivity with the highly homologous family member AGR3 at concentrations up to 500 ng/ml (Figure 3A). Furthermore, the AGR2 standard curve was not significantly affected by co-addition of equivalent amounts of AGR3, demonstrating that the presence of AGR3 does not interfere with the current ELISA format (Figure 3A). The present ELISA format produced a reproducible and linear dose–response curve in the range 78–5000 pg/ml of rhAGR2 standard (Figure 3B). Intra-assay variation determined by repeated (n = 5)
Figure 2 Identification of AGR2 in ovarian cancer patient plasma by MS

(A) Experimental masses observed following MALDI–TOF/TOF analysis of trypsin-digested fractions of ovarian cancer patient plasma obtained following immunoprecipitation with the anti-AGR2 MAb. Masses shown in bold type were confirmed and sequenced using ESI–MS/MS. The theoretical mass, missed cleavages and any methionine oxidation for each observed peptide are shown. The corresponding sequence and peptide position within the AGR2 precursor protein is given. (B) Sequence coverage of AGR2 precursor protein (Swiss-Prot O95994) obtained by MS analysis of immunoprecipitated fraction. Note that the N-terminal 20 amino acids shown in italics represent the signal sequence presumably cleaved from the mature secreted form.

measurement of 156 and 2500 pg/ml rhAGR2 standards was shown to be 4.2% and 1.5%, respectively. Inter-assay variation was assessed at five rhAGR2 concentrations (ranging from 312 to 5000 pg/ml) in five repeat assays producing an overall mean coefficient of variation of 5.5% (range 11.2% for 312 pg/ml standard to 1.5% for 5000 pg/ml standard). Mean absorbance of 100 pg/ml rhAGR2 standard was significantly greater than blank (0.40 ± 0.040 compared with 0.31 ± 0.026; values are means ± S.D., n = 4; P = 0.009) and was close to ± 2 S.D. of mean blank absorbance, suggesting that the limit of detection was close to 100 ng/ml. Preliminary studies using rhAGR2 (16 and 32 ng/ml) spiked into normal human plasma showed that recovery as measured by ELISA was 60 ± 19% and 97 ± 13% (values are means ± S.E.M., n = 2 or 3 respectively). Serial dilution (neat to 1:4) of an expressing AGR2 plasma sample demonstrated a linear dose–response relationship (results not shown), demonstrating that accurate interpolation from the standard curve can be expected over a broad concentration range.

Compared with normal healthy controls, median plasma concentrations of AGR2 were significantly higher for all (stages I–III) ovarian cancer cases taken together (P < 0.001) and also when considering only early stage (stages I–II) patients (P < 0.001) (Figure 4A). When plasma AGR2 levels for the individual cancer stages were compared with those of normal controls (Figure 4B), the median plasma AGR2 concentrations from patients with stages II and III were shown to be significantly higher than normal controls (P < 0.001 and P < 0.05, respectively), whereas median AGR2 concentrations in plasma from patients with stage I were approximately twice the concentration of controls but did not reach statistical significance in this small sample set. By comparison, CA125 was measured in parallel, and the median level was shown to be significantly (P < 0.01) increased in the circulation of all ovarian cancer cases (stages I–III) and to progressively increase from stage I to III carcinomas (Figures 4C and 4D).

All ovarian tumour types analysed exhibited elevated median circulating levels of AGR2, and both serous and non-serous cases of all stages demonstrated significantly increased (P < 0.001) median concentrations of plasma AGR2 compared with normal controls (Figures 5A and 5B). In contrast, although median circulating plasma CA125 concentrations were significantly elevated in both serous and non-serous ovarian tumour groups (P < 0.001 for each group compared with control), median CA125 levels tended to be higher in serous cases compared with non-serous cases (Figure 5C). When the non-serous tumour group was divided into the constituent tumour types (mucinous, endometrioid, clear cell and mixed mullerian type), the number of samples in each group were too low to allow meaningful statistical analysis; however, the present results suggest that circulating AGR2 levels are particularly elevated in several non-serous cancer patients that display a low CA125 concentration (Figures 5B and 5D). There was no significant correlation across all ovarian cancer cases between circulating levels of CA125 and AGR2.
In the present study, we have used a sandwich ELISA and MS to demonstrate, for the first time, the presence of AGR2 protein in human plasma and show that it is significantly increased in the plasma of ovarian cancer patients. The increase in circulating AGR2 concentrations in ovarian cancer cases showed no significant correlation with an existing tumour marker CA125, and this was particularly evident when serous and non-serous ovarian cancer cases were compared. In comparison with an existing tumour marker, CA125, which is known to be more highly expressed in serous compared with most non-serous cancers [14], median plasma concentrations of AGR2 were increased similarly in these two groups, suggesting that circulating AGR2 levels may complement the diagnostic performance of CA125. Consistent with this, comparison of ROC curves for CA125 alone with the combination of CA125 and AGR2 showed a significant increase in the AUC for the combined markers, suggesting enhanced ability to discriminate between case and control subjects. Several groups have reported promising data showing that the use of multibiomarker tests that include CA125 can deliver enhanced sensitivity and specificity over the performance of CA125 alone [15]. The present results suggest that measurement of plasma AGR2 concentrations in ovarian cancer patients may be of potential benefit as part of a multibiomarker panel, particularly in combination with CA125.

Immunohistochemical staining of ovarian tissues in the present study showed that normal ovarian surface epithelium displays little or no detectable AGR2; however, the majority of ovarian epithelial carcinoma specimens display moderate to strong staining for AGR2, suggesting that the transformed ovarian epithelium may contribute to the increase in circulating levels of AGR2. Collectively, our results are consistent with previous gene array studies that have shown that AGR2 mRNA levels are increased in ovarian tumours of various histological types and may be expressed at proportionately higher levels in non-serous tumours [9,10].

Several immunohistochemical studies have demonstrated increased expression of AGR2 in other human cancers, including breast, prostate and pancreatic carcinoma tissue [4,5,7], and additional studies showed that AGR2 could be detected in conditioned medium from human prostate and pancreatic tumour cell lines [5,7,16]. Taken together, these findings may imply that overexpression of AGR2 is widespread in many human carcinomas and may be of limited value as a plasma biomarker specifically for ovarian cancer as opposed to other cancers. Although AGR2 appears to be secreted from some pancreatic and prostate cancer cell lines in vitro [5,7,16], there have been no reports of detectable levels of circulating AGR2 protein in other cancer patients. Riener et al. [17] recently
Figure 4  Comparison of AGR2 and CA125 concentrations in plasma of control and cases with various stages of ovarian cancer
Circulating levels of AGR2 and CA125 were compared between control, all ovarian cancer cases and early stage (I and II) cases (A and C) and between stages I, II and III (B and D). Note that there were no patients with stage IV disease available. Open circles indicate the individual values of each AGR2 or CA125 sample, and horizontal lines show the median concentration of each group. *P < 0.05 and **P < 0.01 compared with control (Kruskal–Wallis test, followed by Dunn’s multiple comparison test).

Figure 5  Comparison of AGR2 and CA125 concentrations in plasma of control and ovarian cancer cases of various tumour types
Circulating levels of AGR2 and CA125 were compared between control, serous and non-serous ovarian cancer cases (A and C). Further comparison was made across all ovarian cancer types tested (B and D). Open circles indicate the individual values of each AGR2 or CA125 sample, and horizontal lines show the median concentration of each group. **P < 0.01 compared with control (Kruskal–Wallis test, followed by Dunn’s multiple comparison test). Endo, endometrioid carcinoma; CC, clear cell carcinoma; MMT, mixed Mullerian type carcinoma.
Figure 6 ROC curve analysis comparing performance of CA125 and AGR2 alone with combined CA125 and AGR2 for the ability to distinguish between control and ovarian cancer cases

ROC curves and AUC for AGR2 and CA125 alone and for CA125 in combination with AGR2 are shown. All three curves showed significant ability to discriminate between control and ovarian cancer patients ($P < 0.0001$). Comparison of the curves demonstrated that the combination of CA125 + AGR2 displayed a significantly greater AUC than using CA125 alone ($P < 0.05$).

showed that immunostaining of AGR2 in pancreatic ductal adenocarcinoma was significantly higher in female patients, suggesting the possibility of gender-dependent differential expression. Furthermore, in the prostate cancer cell line PC3, a subclone that was shown to be resistant to docetaxel expressed and secreted 2.4 times less AGR2 than the parent cell line, suggesting that the rate of secretion of AGR2 from tumour cells with different phenotypes can vary considerably [16].

It has been previously shown that circulating tumour cells derived from prostate, breast and gastrointestinal tumour patients with advanced metastatic disease expressed high levels of AGR2 mRNA, and, in gastrointestinal tumours in particular, AGR2 may provide a valuable surrogate marker of circulating tumour cells [18,19]. However, the previously published results for these other cancers detected the mRNA for AGR2 using quantitative PCR techniques, which benefit from high sensitivity, but are not readily amenable to routine diagnostic/prognostic tests. Whether or not these tumour types secrete measurable levels of AGR2 protein into the circulation remains to be determined.

An unequivocal functional role for AGR2 in carcinogenesis has not been established; however, several studies have implicated AGR2 as a factor involved in tumour cell growth and survival [6–8]. Overexpression of AGR2 in a benign non-metastatic mammary tumour cell line led to lung metastasis when cells were transferred to the mammary glands of syngeneic hosts, suggesting a role for AGR2 in the process of metastatic spread of tumour cells [4]. Furthermore, conditioned medium from MPanc-96 pancreatic tumour cells in which AGR2 expression had been silenced showed significantly reduced capacity to stimulate proliferation of pancreatic tumour cells in vitro [7], implying that secreted AGR2 may have paracrine or endocrine growth-promoting actions on other cells.

Taken together, the present study demonstrates that AGR2 protein is expressed in neoplastic ovarian epithelium, is released into the circulation and can be detected using the well-established diagnostic/prognostic ELISA technique. Furthermore, the present experiments show that a blood test for AGR2 protein, in combination with CA125, may potentially aid detection of a subset of otherwise undetectable non-symptomatic ovarian carcinomas.

Additional larger studies are needed to determine the biological significance of elevated levels of circulating AGR2 associated with ovarian cancer and to validate the prognostic value in subsets of ovarian carcinoma patients.

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