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Original Article

A multiplex serum protein assay for determining the probability of colorectal cancer

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Abstract: Our purpose is to develop a serum assay to determine an individual's probability of having colorectal cancer (CRC). We have discovered a protein panel yielding encouraging, clinically significant results. We evaluated 431 serum samples from donors screened for CRC by colonoscopy. We compared the concentration of seven proteins in individuals with CRC versus individuals found to be CRC free. The assay monitored a single peptide from each of seven proteins. Comparing CRC to normal samples in univariate two-sample t-tests, 6 of the 7 proteins yielded a p-value less than 0.01. Logistic regression was used to construct a model for determination of CRC probability. The model was fit on a randomly chosen training set of 321 samples. Using 6 of the 7 proteins (ORM1, GSN, C9, HABP2, SAA2, and C3) and a cut point of 0.4, an independent test set of 110 samples yielded a sensitivity of 93.75%, a specificity of 82.89% and a prevalence-adjusted negative predictive value (NPV) of 99.9775% for the assay. The results demonstrate that the assay has promise as a sensitive, non-invasive diagnostic test to provide individuals with an understanding of their own probability of having CRC.

Keywords: Colon cancer, proteomics, cancer, colon, mass spec, MRM, colorectal, CRC

Introduction

CRC is highly curable when diagnosed at an early stage, with a 90% five-year survival rate according to the Colon Cancer Alliance (Colorectal Cancer Statistics update 2011). The U.S. Preventive Services Task Force recommends screening for CRC using high-sensitivity fecal occult blood testing (FOBT) (sensitivity 64.3% (95% CI = 35.6% to 86.0%), specificity 90.1% (95% CI = 89.3% to 90.8%) for detecting cancer) [1], sigmoidoscopy, or colonoscopy beginning at age 50 and continuing until age 75. However, a 2008 report from the Center for Disease Control established that in the United States only 60% of adults age 50 or older had undergone a sigmoidoscopy or colonoscopy within the previous 10 years or had used a FOBT home test kit within the preceding year according to Centers for Disease Control and Prevention. This low compliance rate has been attributed to the time and cost associated with sigmoidoscopy or colonoscopy as well as modesty, fear of pain, and an unwillingness to handle fecal specimens [2]. In addition, the miss rates of colonoscopy and sigmoidoscopy reflect poor adherence to a necessary day-long bowel preparation procedure. A recent study involving 12,787 individuals reported that improper bowel preparation prior to colonoscopy resulted in a miss rate of 42% [3].

As a result, a number of research and development efforts are now focused on biomarkers for use in assays based upon non-invasive samples, such as serum, to evaluate the probability of CRC. Such assays would not necessarily replace invasive or unpleasant procedures, but would provide individuals and physicians with information on which to base a decision to either have or defer such procedures. Several serum CRC biomarkers have been reported, but none has demonstrated sufficient sensitivity and specificity to displace FOBT or fecal immuno-chemical test (FIT) as a screening test [4].

Previously, we conducted a biomarker discovery project to identify serum proteins differentially

Table 1. Summary of cohort

Category	Normal	CRC	Two-Sided p-Value (a)
Total	259	172	
Female [n (%)]	89 (34.4%)	93 (54.4%)(b)	< 0.0001
Male [n (%)]	170 (65.6%)	78 (45.6%)(b)	
Age Range	43-71	30-82	
Mean Age (Std. Dev.)	56.8 (4.75)	61.6 (10.83)	< 0.0001
Stage I/II/III/IV		19/53/71/27(c)	
Smokers [n (%)]	59 (22.9%)(d)	31 (18.0%)	0.2263
Location of tumor			
Ascending Colon		9	
Cecum		12	
Cecum sigmoid		1	
Colon		1	
Descending Colon		5	
Hepatic Flexure		12	
Rectosigmoid		3	
Rectum		69	
Sigmoid		50	
Splenic Flexure		3	
Transverse Colon		7	

(a) The two-sided p-value is based on a two-sample test of equality between normal and CRC samples. (b) One CRC sample did not have a gender reported. (c) One CRC sample was from a patient with a reoccurrence of cancer, and for another, no stage was provided. (d) One normal sample did not have a smoking status reported.

expressed in normal versus CRC serum samples. During the discovery studies these proteins were identified by mass spectrometry methods and stringent criteria for the identification were applied (two or more peptides per protein and a false discovery rate of less than 1%). Commercially available immunoassays were not available for many of the proteins found, and thus, a mass spectrometry assay method was selected. Initially 46 peptides from 14 proteins were included in the assay, but the peptides reported here provide the model with the best sensitivity, specificity and negative predictive value.

Materials and methods

Sample set

All 431 samples were obtained from ProteoGenex Inc. Patients were recruited at a gastroenterology unit in Moscow, Russia from an average-risk screening population and underwent a colonoscopy. The research protocol was reviewed and approved by the appropriate ethics committees and all participants gave written informed consent. Samples were collected in two centers. Normal control samples were drawn at the gastroenterology unit 3 to 30 days after colonoscopy and CRC samples were drawn at the oncology surgery center 5-90 days before surgery. Approximately 15 mL of blood was collected in SST Tubes (Greiner, Cat. #454067) and processed in a central laboratory according to the tube manufacturer's directions. Samples were aliquoted into 1mL cryotubes and frozen at -80C within 6 hours of collection. Samples were shipped to the sponsors testing laboratory on dry ice.

The size and location of each lesion were recorded. A pathologist examined each surgical resection specimen on site to determine the diagnosis and the respective staging.

CRC samples were drawn between September 3, 2009 and December 8, 2010. Normal control samples were drawn between March 16, 2010 and January 28, 2011. During the overlap draw period defined by the period starting with the first normal sample draw date and ending with the last CRC sample draw date (March 16, 2010 through December 8, 2010) 290 samples (67%, 93 CRC (54% of total CRC) and 197 normal (76% of total normal)) were drawn. Seventy nine CRC samples were drawn prior to the overlap period and 62 normal samples were drawn after the overlap period. Samples from the overlap period were included in both the training and test sets. **Table 1** provides a summary of

Table 2. Summary of the proteins used in the assay

Protein name (gene name)	Accession code (Gene ID)	Protein function	Peptide monitored	Standard curve (ng/ml)	Regression coefficient (final model)
Alpha-1-acid glycoprotein 1 (ORM1)	P02763 (5004)	Acute-phase reactant, increases due to acute inflammation (a)	SDVVYTDWK	4.9-1250	-0.5259
Gelsolin (GSN)	P06396 (2934)	Also known as brevin, or actin-depolymerizing factor; principal intracellular and extracellular actin-severing protein (b) [5,6]	IFVWK	9.8- 625	-0.6526
Complement component C9 (CO9)	P02748 (735)	Final component of the complement system, participates in the formation of the Membrane Attack Complex (MAC) (c)	AIEDYIN-EFSVR	78.1-2500	0.9400
Serine peptidase inhibitor, clade A, member 3 (SERPINA3)	P01011 (12)	Member of serine protease inhibitors (serpins) protein superfamily, inhibitory activity against proteases, associated with inflammatory reactions, putative marker for Alzheimer's disease, malignant melanoma and gastric cancer (d), expression in CRC [7,12]	ADLSGITGAR	9.8- 625	NA
Hyaluronan-binding protein 2 (HABP2)	Q14520 (3026)	An extracellular serine protease that binds hyaluronic acid and is involved in cell adhesion (e)	VVLGDQDLK	2.4 – 310	1.9734
Serum amyloid A protein (SAA2)	P02735 (6289)	Up regulated by a variety of inflammatory stimuli, including cytokines and glucocorticoids, elevated systemic concentrations of both SAA and tumor necrosis factor-alpha are a feature of inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease(f) [8]	FFGHGAED-SLADQAANE WGR	9.8-1250	0.6726
Complement component C3 (CO3)	P01024 (653879)	Plays a central role in the activation of complement system, activation is required for both classical and alternative complement activation pathways (g).	IHWESASLLR	9.8-1250	-0.3059

(a) National Center for Biotechnology Information (NCBI), ENTREZ, ORM1 orosomucoid 1 [*Homo sapiens*],update August 2011, http://www.ncbi.nlm.nih.gov/gene/5004. (b) NCBI, ENTREZ, GSN gelsolin [*Homo sapiens*], update August 2011, http://www.ncbi.nlm.nih.gov/gene/2934. (c) NCBI, ENTREZ, C9 complement component 9 [*Homo sapiens*], update Aug 2011, http://www.ncbi.nlm.nih.gov/gene/735. (d) NCBI, ENTREZ, SERPINA3 serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 [*Homo sapiens*], update August 2011, http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Retrieve&dopt=full_report&list_uids=12. (e) NCBI, ENTREZ, HAPB2 hyaluronan binding protein 2 [*Homo sapiens*], update Aug 2011, http://www.ncbi.nlm.nih.gov/gene/3026. (f) NCBI, ENTREZ, SAA2 serum amyloid A2, update Aug 2011, http://www.ncbi.nlm.nih.gov/gene/6289. (g) NCBI, C3 complement component C3 [*Homo sapiens*], update 9-Oct 2011, http://www.ncbi.nlm.nih.gov/gene/718.

the samples included in the study.

Sample analysis

Serum was diluted 25 fold in deionized water, reduced, alkylated, and digested with trypsin, then prepared for analysis by mass spectrometry. Each preparation provided enough volume to analyze each sample twice (analytical replicates). The peptide WHWLQL (T2903, Sigma Aldrich) was used as an internal standard (IS) and was added to each sample before sample processing. Technicians were blinded to sample status. The 431 samples were randomly divided into six batches for processing. Each batch had approximately the same number of normal and CRC samples.

The assay method was high performance liquid chromatography (HPLC) coupled with multiple reaction monitoring tandem mass spectrometry (MRM LC/MS/MS), which specifically monitors the proteolytically-generated peptides of the seven proteins. Peptides were selected based on reproducibility of results from earlier experiments in combination with results of statistical analysis conducted on the results from these experiments. The proteins and peptides included in the assay are listed on **Table 2**.

These samples were analyzed using a triple quadrupole API 5000 mass spectrometer (Applied Biosystems) equipped with the 10 ADVP HPLC system (Shimadzu Scientific Instruments) using a reverse phase column (Varian

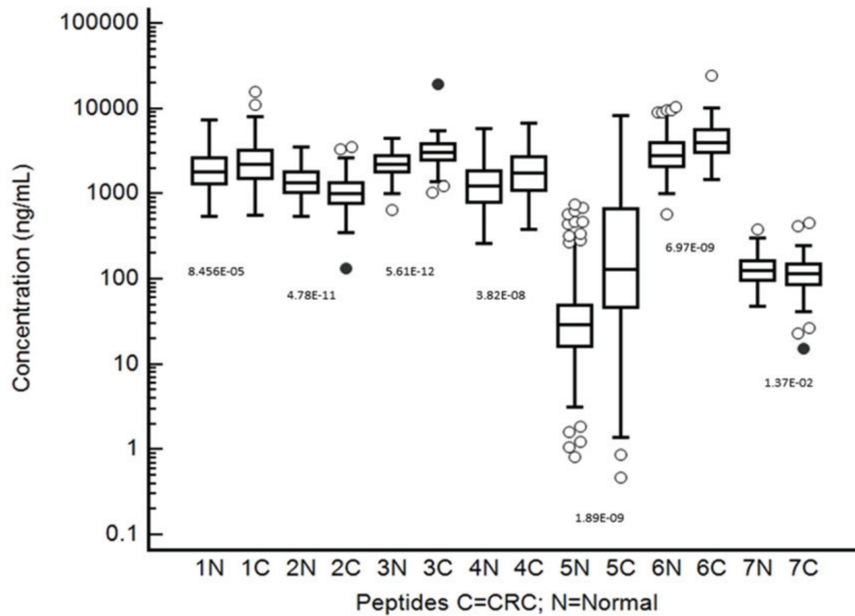

Figure 1. Peptide concentration ranges. Proteins are abbreviated as follows: 1 = ORM1, 2 = GSN, 3=C9, 4 = SERPINA3, 5= SAA2, 6 = C3 and 7 = HABP2). The median value is the line within the box. The p-value comparing the normal (N) and CRC samples is the number below each pair of box plots.

MetaSil AQ C18, 50x2 mm).

The standard curves were made using the heavy-isotope version of each peptide and the same IS that was added to the samples [9]. The concentration ranges for the standard curves are provided in Table 2. For quality assurance, standard curves were required to have a correlation coefficient greater than 0.98. Quality control (QC) serum samples were used to evaluate the intra and inter batch variation. Intra-batch variation of three QC samples were required to have a coefficient of variance (CV) of less than 20%, and inter-batch variation was evaluated using a Q test on the QC0 samples. Replicate concentration values for each peptide in each sample were averaged.

Statistical analysis

An unpaired two-sample t-test was performed on each peptide comparing the concentration data from the normal samples to the concentration data from the CRC samples. To assess the performance of these peptides in a multivariate setting, logistic regression was performed using SAS® Version 9.2 (SAS Institute, Cary, NC) in order to model the probability of CRC. Only the main effects of each peptide were considered in the modeling process; no interactions between peptides were included. Stepwise selection was used to determine which peptides should be included in the model. A significance level of 0.05 was required for entrance into the model based on the score chi-squared test, and a significance level of 0.05 was required for removal from the model based on the Wald test.

Once the model was fit on the selected peptides, a cut-point of 0.4 was chosen for the assay because it produced the highest combination of sensitivity and specificity in the training set. A binary qualitative assay result was generated (i.e., presence or absence of CRC). Samples with a probability of CRC value less than or equal to 0.4 were given a test result of “negative” for CRC (which may include some false negatives), and all samples with a probability of CRC value above 0.4 were given a test result of “positive” for CRC (which may include some false positives). The result of the assay in the test set was used with the known status of each sample to compute sensitivity and specificity as well as prevalence-adjusted (0.31%) NPV.

Principle Component analysis (ArrayTrack, FDA) was used to evaluate data from normal and CRC samples collected during different time intervals.

Results

Concentration data for the seven proteins

Table 2 lists the proteins in the assay and provides information about the function of the proteins as well as the specific peptide monitored for each protein. The proteins are abundant serum proteins [10] whose concentrations have been reported to be altered during the acute

phase. Figure 1 is a box plot which provides the concentration range found and the p-value based on unpaired two-sample t-tests conducted for each peptide, comparing normal and CRC samples. Although the p-values were low ($p<0.001$) except for HABP2, it is clear from this figure that no single peptide is sufficient to separate CRC samples from normal samples because there is overlap in the concentration values between normal and CRC samples for all peptides.

The samples were collected over a 16 month period. The collection of CRC samples started 6 months before the collection of normal samples and the collection of normal samples continued for about a month after collection of CRC samples ended. In order to evaluate if these differences in collections times affected the data on samples, PCA analysis was done on all the normal samples and then repeated for all the CRC samples. No subgroups within the normal or CRC samples were found suggesting that the collection times had no effect on the results.

Statistical model

The data were randomly divided into a training set and a test set. The training set consisted of approximately 75% (321/431) of the samples. Logistic regression analysis with stepwise selection on the training set resulted in the selection of six of the seven peptides. Table 2 provides the regression coefficients for each peptide in the model. One of the proteins selected by the model, HABP2, had a large p-value ($p=0.0137$) based on the univariate t-test while the excluded protein, SERPINA3, had a small p-value ($p=3.82E-08$), providing evidence for covariation of certain peptides and independence for others.

For the final model, the area under the curve (AUC) of the Receiver Operator Characteristic (ROC) [11] curve was 0.9003 for the training set (Figure 2). Model fit was assessed using Hosmer and Lemeshow's Goodness-of-Fit test. The non-significant p-value ($p=0.1250$) indicates that there is no evidence of poor model fit.

The test set was then assessed using the model. For each sample in the test set, the model produced an estimate of the probability of CRC. The ROC curve of the model relative to the test data had an AUC of 0.8989 (Figure 3).

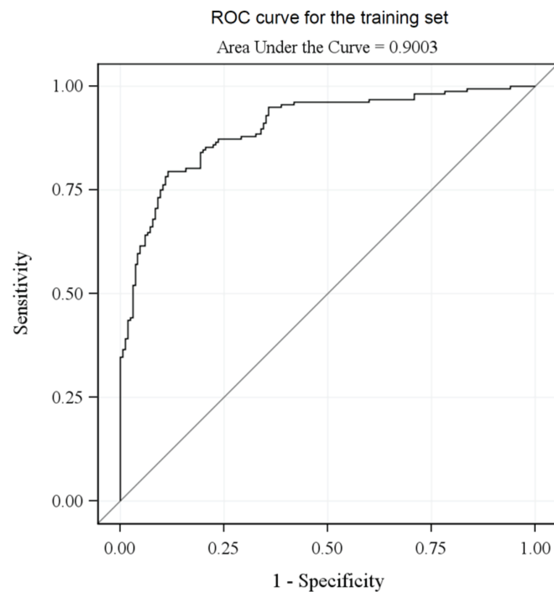

Figure 2. ROC curve for the training set. The tradeoff between sensitivity (true positive) and specificity (true negative) for CRC and normal samples at every possible cut-off is illustrated. The AUC measures discrimination, which is the ability of the final model to correctly classify normal and CRC samples. The diagonal line represents no ability to differentiate between the two groups (AUC=0.50) and a perfect test would have AUC=1.0.

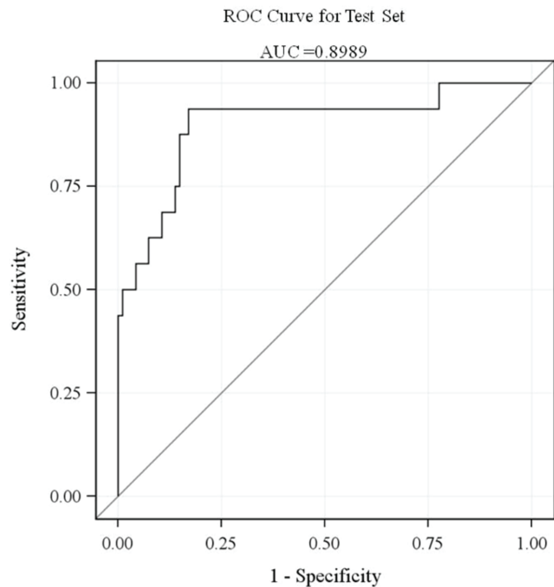

Figure 3. ROC curve for the test set. This figure displays the tradeoff between sensitivity (true positive) and specificity (true negative) for CRC and normal samples.

Table 3. Influence of stage of disease

Cancer Stage	Training set		Test set	
	N	Proportion predicted to have cancer(a) (95% CI)	N	Proportion predicted to have cancer(a) (95% CI)
I	17	0.71 (0.44, 0.90)	2	1.00 (0.16, 1.00)
II	50	0.82 (0.69, 0.91)	3	1.00 (0.29, 1.00)
III	64	0.86 (0.75, 0.93)	7	0.86 (0.42, 1.00)
IV	23	0.96 (0.78, 1.00)	4	1.00 (0.40, 1.00)

(a) Proportion of CRC subjects in the training and test who had a model score >0.4.

In the test set, when using the model cut-point of 0.4, the final assay had a sensitivity of 93.75% (95% CI, 69.77 - 99.84), specificity of 82.98% (95% CI, 73.84 – 89.95) and a prevalence-adjusted NPV of 99.9775% for the prediction of CRC.

Sample demographics

Table 1 summarizes the demographics represented in the 431 samples, 259 of which were normal samples and 172 of which were CRC samples. Due to the slight inequality of age and gender distributions among the CRC and normal samples, the influence of age and gender on the probability of having CRC was determined using two-sample t-tests of equal means. The average probability of CRC samples below the median age of the CRC group (*i.e.,* age < 61.5) was 0.7445 and the average probability of CRC samples above the median (age ≥ 61.5) was 0.7279 (p=0.6978), indicating that there was no significant influence of age on the probability of CRC. Similarly, for normal samples, there was no significant difference between samples from subjects below the median age of the normal group (age < 57.0) *vs.* samples from subjects above the median age (0.2421 *vs.* 0.2494, p=0.7826). In a similar comparison, there was no difference between male and female assays scores in the CRC group (0.7248 *vs.* 0.7443, p=0.6536) or in the normal group (0.2493 vs. 0.2403, p=0.7441). Importantly, both groups had similar percentages of smokers.

Influence of disease stage on assay results

Table 3 provides a summary of the samples divided by the stage of disease. The sensitivity improved by 25% from Stage I samples (71%) to Stage IV samples (96%) in the training set. In the test set, the probability of CRC was above 0.4 for all but one CRC sample, with the only misclassification occurring in one Stage III sample. Two CRC samples in the training set could not be classified according to their stage (one CRC sample was from a patient with a reoccurrence of cancer, and for another, no stage was provided). These two samples were excluded from Table 3.

Influence of other chronic diseases

Samples from individuals with other disease conditions commonly found in screening populations were not excluded from the training or test sets. Several proteins in the assay have known association with inflammatory response, Table 2. Of the 165 normal samples in the training set, 116 were from individuals with a chronic disease, which was similar to the chronic disease rates in the test set (63 out of 94). To test the specificity of the assay in subjects with chronic disease and inflammation we compared the number of false positives detected in the test set in individuals with a chronic disease to the number of false positives in individuals without any chronic disease. The false positive rate is similar between the two groups: 6 out of 31 (0.19; 95% CI, 0.07-0.37) from individuals without chronic disease and 10 out of 63 (0.16; 95% CI, 0.08-0.27) from individuals with chronic disease. A test of equal false positive rates between the two groups confirms that there is no statistically significant difference between individuals with chronic disease and those without chronic disease (p-value = 0.6728).

Discussion

The current standard of care for detection of CRC in individuals over the age of 50 is colonoscopy. The compliance rate, however, is only

about 60%, despite the fact that early detection typically results in better patient outcomes [13]. And, colonoscopy miss-rates can exceed forty percent when patients do not comply with necessary bowel preparation procedures. This CRC serum assay is intended to provide individuals with important information regarding their own probability of having CRC, especially those who choose not to have a colonoscopy, which may in turn encourage individuals with a high probability of CRC by this assay to undergo a colonoscopy. When the probability of CRC is in the mid range for the assay (0.3-0.6), the patient's decision will reflect his or her individual risk tolerance.

Because of the limitations of present screening methods, including colonoscopy and FOBT, there are several efforts underway to identify, confirm and validate serum biomarkers which could be used for routine CRC screening during annual physicals [4, 5, 14-17]. Over the past several years, we have conducted such studies and have developed our protein-based serum assay, which simultaneously measures one peptide for each of seven proteins in serum. The tryptic peptides chosen for the assay are unique to the proteins being measured and are quantified using as little as 0.01 mL of serum. MRM LC/MS/MS cycle time is 4.5 minutes from the start of one sample to the start of the next sample, and sample preparation is relatively simple making the assay suitable for high-throughput techniques and broad implementation.

In this study we evaluated 431 samples, including 172 drawn from patients with CRC. Six of the seven peptides in the assay had an average concentration that was greater in CRC samples than in normal samples. This result mirrors the results from our discovery experiments as well as the data obtained for samples used during the development of the assay (unpublished results). One of the seven proteins, GSN, had a lower average concentration in CRC samples than in normal samples, as has been reported by others [5]. None of the individual proteins provided high enough sensitivity to act as a single biomarker to determine the probability of CRC in a screening test. In our multivariate analysis we developed a model to achieve our goals of high sensitivity, 93.75% (95% CI, 69.77 - 99.84) and high specificity, 82.98% (95% CI, 73.84 – 89.95) with a prevalence adjusted NPV of 99.9775 % in the randomly chosen 110 sample test set.

The assay produces a probability of CRC as a number ranging from 0 to 1, the higher the number, the greater the probability of CRC. The 95% confidence interval around any probability calculated using the assay had a median half-width of 0.0968. A cut-off number (0.40) was chosen for the purpose of reporting the sensitivity, specificity and NPV of the model. Importantly, the probability of CRC determined by the assay does not correlate with age, gender or comorbidity, only stage of disease.

Because the samples analyzed in this study were from a single cohort, the results reported here will be further validated in a large multicenter prospective trial. Once successfully validated, this assay could be performed in parallel with other routine tests drawn during annual physical examinations. Results from this assay would provide physicians and patients with important information that could be used to assist in the decision as to the need for either an immediate colonoscopy or follow-up screening tests at established intervals.

Acknowledgments

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