Black raspberries inhibit N-nitrosomethylbenzylamine (NMBA)-induced angiogenesis in rat esophagus parallel to the suppression of COX-2 and iNOS

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Angiogenesis, the formation of new blood vessels, is critical to tumor growth and metastasis. Vascular endothelial growth factor (VEGF), an important angiogenic activator, is essential for angiogenesis. Our laboratory has used a rodent model of human esophageal squamous cell carcinoma (ESCC) to identify putative chemopreventive agents for this disease and determine their mechanisms of action. We reported that dietary black raspberry powder (BRB) inhibits N-nitrosomethylbenzylamine (NMBA)-induced tumor development in the rat esophagus by inhibiting the formation of DNA adducts and reducing the proliferation rate of preneoplastic cells. On a molecular level, BRB downregulates the expression of c-Jun, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). In this study we analyzed the effect of BRB on angiogenesis. VEGF expression was determined by real-time RT–PCR and immunohistochemical analysis of microvessel density (MVD). BRB significantly suppressed VEGF-C expression from a 2.38 (± 0.34)-fold increase in animals treated with NMBA alone to a 1.08 (± 0.22)-fold increase in animals treated with NMBA plus BRB ($P < 0.005$). The MVD of esophagus was decreased from 53.7 ± 5.6 vessels/cm in animals treated with NMBA alone to 22.6 ± 2.6 vessels/cm in animals treated with NMBA plus BRB ($P < 0.0001$). Our data also suggest that downregulation of VEGF is correlated with suppression of COX-2 ($r^2 = 0.86, P < 0.001$) and iNOS ($r^2 = 0.81, P < 0.005$). As high vascularity is a risk factor for metastasis and tumor recurrence, BRB may have cancer therapeutic effects in human esophageal cancer.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal gastrointestinal malignancies worldwide (1). The incidence rate of this disease varies dramatically in different geographical areas; China has the highest rate, with ~250,000 new cases diagnosed yearly, accounting for half of the world’s new cases (2). Risk factors for ESCC include tobacco use, alcohol consumption, nutritional deficiency and intake of food contaminated with various mycotoxins. ESCC grows more quickly than other kinds of gastrointestinal malignancies and patients with this disease have a very poor prognosis. The major causes of death are hematogenous metastasis to liver and lung and lymph metastasis (3). Although surgery, chemotherapy and radiotherapy alone or combined are used, the overall 5 year survival rate for this disease is still very low, ranging from 5 to 15%.

Angiogenesis, the formation of new capillaries from preexisting blood vessels, was first defined by Shubik in 1968 (4). It is essential for the tumor growth and expansion because it enhances the opportunities for tumor cells to reach the general circulation and metastasize. Solid tumors <2 mm in diameter can obtain oxygen and nutrients from neighboring blood vessels by simple passive diffusion. Beyond this size, new vasculature is required for tumor cell growth and survival (5). Several growth factors were reported to have angiogenic activity including vascular endothelial growth factor (VEGF), basic fibroblast growth factor and platelet-derived growth factors (6). Unlike other growth factors, VEGF is a highly specific mitogen for vascular endothelial cells, inducing their proliferation and migration. VEGF, therefore, is regarded as the major angiogenesis factor during carcinogenesis and tumor metastasis. The VEGF family includes VEGF-A, -B, -C, -D and -E (7). The different regulation and tissue distribution of these family members suggest that they have different roles in angiogenesis. VEGF-A is overly expressed in human colon cancer (8). VEGF-C is upregulated in various human cancers—lung, breast, head and neck, thyroid, stomach, uterus, prostate, colon, and ESCC (9–17). VEGF-D expression is elevated in human colorectal cancer (18).

Microvessel density (MVD) in histological sections is commonly assayed to estimate the degree of new blood vessel formation. Immunohistochemistry is performed to highlight blood endothelial cells using antibodies against either platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31), CD34, CD36, CD105, Ulex europaeus agglutinin 1 or von Willebrand factor (19,20). Numerous studies demonstrate a positive correlation between VEGF expression and MVD in human ESCC (21).

Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are two critical inducible enzymes that are elevated in many human cancers including ESCC (22,23). Their metabolites, prostaglandin E$_2$ (PGE$_2$) and nitric oxide (NO), can affect cell proliferation, differentiation and angiogenesis (24). The expression of genes for VEGF, COX-2 and iNOS are correlated in human lung, colon, prostate, pancreas and gastric cancers (25–29). To our knowledge, no study has assessed the relationships among VEGF, COX-2 and iNOS in human ESCC.
Our laboratory has been working on chemoprevention of esophageal cancer for more than 20 years using pure synthetic compounds and natural food products. A series of studies in our laboratory demonstrated that dietary black raspberries have many protective effects in a rodent animal model of carcinogen-induced ESCC through reducing DNA adduct formation, decreasing preneoplastic cell proliferation and inhibiting the expression and activity of COX-2 and iNOS (30,31). However, the anti-angiogenic potential of dietary black raspberry powder (BRB) has never been evaluated.

The present study was designed to retrospectively examine the expression of VEGF-C and -D and MVD in esophagus tissue from rats treated with N-nitrosomethylbenzylamine (N MBA) alone or NMBA plus dietary BRB. These tissues were collected from a previous chemoprevention study in which rats fed BRB had 41% fewer tumors than rats fed a regular diet after exposure to NMBA (31). In addition, to contribute to our understanding of the mechanism of inhibition of VEGF by BRB, we determined whether there were correlations between VEGF and COX-2, VEGF and iNOS, and COX-2 and iNOS expressions in the esophagus from rats treated with NMBA alone versus NMBA plus dietary BRB.

Materials and methods

Chromatin and reagent kits
TRIZol reagent was obtained from Gibco BRL (Gaithersburg, MD). The QuantiTect SYBR Green RT–PCR Kit was purchased from Qiagen (Valencia, CA). Primers for VEGF-C, VEGF-D and the housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT) were synthesized by Life Technologies (Gaithersburg, MD). CD34 antibody was obtained from BioGenex (San Ramon, CA).

Animal treatments and sample collections
Esophageal samples for the current investigation of angiogenesis were obtained from a previous chemoprevention study (31). In brief, 125 male Fischer-344 rats, aged 4–5 weeks, were divided into three groups of 25–50 animals each and treated s.c. with either NMBA (0.25 mg/kg body weight) or a 1:4 mixture of dimethyl sulfoxide:H2O (the solvent for NMBA) three times per week for 5 weeks. Starting from Week 6, the NMBA-treated rats were fed either regular AIN-76A diet or AIN-76A diet containing 5% dietary BRB. Another group of 25 rats was fed dietary BRB only. At Week 25, rats were fed either regular AIN-76A diet or AIN-76A diet containing 5% BRB or a 1:4 mixture of dimethyl sulfoxide:H2O (the solvent for NMBA) three times per week for 5 weeks. Starting from Week 6, the NMBA-treated rats were fed either regular AIN-76A diet or AIN-76A diet containing 5% BRB. Another group of 25 rats was fed dietary 5% BRB only. At Week 25, 25–50 animals per group were killed and esophageal tumors were counted and sized. The tumor data from this study have been reported (31). One-half of the tissue from each esophagus was collected and frozen immediately in liquid nitrogen and then transferred to a −80°C freezer for subsequent molecular analysis. The other half of each esophagus was fixed in 10% neutral buffered formalin for 4 h and then transferred to phosphate-buffered saline to make paraffin embedded blocks for immunohistochemistry.

RNA isolation and real-time RT–PCR
Total RNA was extracted from frozen esophagi using TRIZol reagent according to the manufacturer’s instructions. All RNA samples were analyzed for integrity of 18S and 28S rRNA by ethidium bromide staining. Total RNA was extracted from frozen esophagi using TRIzol reagent and analyzed for integrity of 18S and 28S rRNA by ethidium bromide staining.

RT was first performed in a GeneAmp 9700 sequence detection system (Perkin-Elmer, Norwalk, CT) using the QuantiTect SYBR Green RT–PCR Kit as recommended by the manufacturer. Primers for VEGF and HPRT were designed according to published sequences with Primer Express Software V 2.0 (Applied Biosystems, Foster City, CA). The primer sequences were VEGF-C, 5'-GGGCCCAAAACGCATTCA-3' (forward) and 5'-CTGTAACATCCAGTTTAGACATGCA-3' (reverse); VEGF-D, 5'-GGTGGAGCGATGGGATGTT-3' (forward) and 5'-GGTGGAGCGATGGGATGTT-3' (reverse) and HPRT, 5'-GTCTGAGATGTCATGAAGGAGAT-3' (forward) and 5'-GGTGGAGCGATGGGATGTT-3' (reverse). RT was first performed at 50°C for 30 min. PCR conditions were 94°C for 15 s, 60°C for 30 s and 72°C for 30 s for 40 cycles. The expression of VEGF-C and -D mRNA was normalized against expression of HPRT. The gene expression was expressed as the amount of change calculated by \( \frac{CT_{\text{treated}} - CT_{\text{control}}} {CT_{\text{control}}} \) where \( CT \) is the PCR cycle number that crossed the threshold, \( \Delta CT \) is calculated as \( CT_{\text{treated}} - CT_{\text{control}} \) and \( \Delta \Delta CT \) is calculated as \( CT_{\text{treated}} - CT_{\text{control}} \), and mRNA levels were calculated as \( (\frac {CT_{\text{treated}} - CT_{\text{control}}}) \) (the mean of triplicates). The expression of VEGF-C and -D mRNA was significantly reduced from a 2.38 ± 0.34-fold increase in rats treated with NMBA; if rats were fed BRB after NMBA treatment, the overall expressed VEGF-C mRNA was reduced from a 2.38 ± 0.34-fold increase in animals treated with NMBA alone to 1.08 ± 0.22-fold.

Immunohistochemistry
The entire esophagus from each animal was cut into three sections as follows: upper, middle and lower. All three sections were embedded on edge in a single block. The blocks were serially sectioned at 4 µm and mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). As described previously (32), paraffin was removed with histoclear and the slides were rehydrated in graded ethanol (100–70%) . Sections were incubated for 20 min incubation with a mouse absorbed link (goat anti-mouse biotinylated immunoglobulin) and a strepavidin–horseradish peroxidase label. The sections were developed with diaminobenzidine chromogen and then counterstained with hematoxylin, dehydrated and mounted. Reagents were supplied by BioGenex.

Determination of MVD
Esophageal MVD was measured by staining sections with antibody specific for CD34 expressed by vascular endothelial cells. Slides were viewed and photographed with a dual-head Nikon microscope with a high-resolution spot camera interfaced with computer-loaded image analysis software (Simple PCI Imaging Systems; Compix, Cranberry Township, PA). The criteria used to identify microvessels in immunostained sections were established by Folkman (4). In brief, the vessel count was performed using ×200 magnification (×20 objective and ×10 ocular). Any brown-staining endothelial cells or endothelial cell clusters outside of adjacent blood vessels, tumor cells or other connective tissue elements were considered a single countable microvessel. The distinct clusters of stained endothelial cells that might be from the same vessel snaking its way in and out of the section were considered distinct and countable as separate microvessels. Vessel lumens were not necessary for a structure to be defined as a microvessel, and red cells were not used to define a vessel lumen. We evaluated the entire esophagus and counted microvessels staining positive for CD34 in all areas including normal epithelium, hyperplasia, dysplasia and papillomas. This procedure allowed the counts to be more representative for each individual esophagus. The total length of each individual esophagus varied from 6 to 10 cm and it was measured to be at the average of 6.5–7.5 cm by ruler. MVD was calculated by dividing the total number of microvessels in each esophagus by the length of the esophagus. Microvessel counts were done independently by two investigators on 10 esophagi per experimental group.

Statistical analysis
All statistical analysis was carried out using GraphPad Prism 4.0. A comparison of differences was analyzed and compared using the Kruskal-Wallis ANOVA followed by Dunnet’s multiple comparison test to identify significant differences among groups when the ANOVA was significant. The Spearman’s correlation coefficient was used to determine any correlation between the expression of VEGF, COX-2 and iNOS mRNA. Differences were considered statistically significant at \( P < 0.05 \). All \( P \)-values were two-sided.

Results
BRB downregulates VEGF mRNA expression
To determine whether dietary BRB has anti-angiogenic effects, both VEGF-C and VEGF-D mRNA were assessed by real-time RT–PCR. VEGF-D mRNA expression in rat esophagus did not change after rats were treated with NMBA. In contrast, VEGF-C mRNA expression was significantly increased in rats treated with NMBA; if rats were fed BRB after NMBA treatment, the overall expressed VEGF-C mRNA was reduced from a 2.38 ± 0.34-fold increase in animals treated with NMBA alone to 1.08 ± 0.22-fold.

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increase in animals treated with NMBA plus BRB (55% reduction, \( P < 0.005 \)) (Figure 1).

**BRB inhibits MVD**

As shown in Figure 2, normal esophageal epithelium contained few capillaries. In rats treated with NMBA alone, the microvessels were more numerous and packed. However, rats fed with NMBA plus BRB had fewer microvessels \((P < 0.0001)\) (Table I).

**Correlations between VEGF, COX-2 and iNOS expression**

We previously found that BRB significantly inhibited COX-2 and iNOS mRNA in NMBA-induced esophageal tumorigenesis (31). Our data here show that BRB significantly suppressed VEGF-C mRNA. To better understand the mechanisms of BRB action, we assessed the correlations between these genes for downregulation by BRB. As shown in Figure 3A, in rats treated with NMBA alone, the expression of VEGF was correlated with the expression of COX-2 but not iNOS. In rats treated with NMBA plus BRB (Figure 3B), however, the expression of VEGF was correlated with the expressions of both COX-2 and iNOS. Moreover, the expressions of COX-2 and iNOS were correlated.

**Discussion**

In this study, we show that dietary BRB significantly inhibited VEGF-C and microvessel formation in NMBA-induced angiogenesis in rat esophagus. Moreover, the downregulation of VEGF-C by BRB is significantly correlated with the modulation of COX-2 and iNOS by BRB. Because angiogenesis plays a critical role in carcinogenesis and VEGF-C is elevated in human ESCC, BRB may have anti-angiogenic potential for treating this disease in humans.

Vascularization is greater in ESCC than in the normal esophageal tissues (33). Esophageal carcinogenesis is a step-wise process with the lesions progressing from normal to hyperplasia, dysplasia and carcinoma. The stage where the ‘angiogenic switch’ is located, however, has not been fully defined. We examined, therefore, MVD in the entire esophagus, including hyperplastic and dysplastic areas, which are considered to be the precancerous lesions.

It is generally accepted that angiogenesis is essential for tumor growth and metastases, which depend on the

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**Table I. Modulation of MVD in esophagus by BRB in NMBA-treated rats**

<table>
<thead>
<tr>
<th>NMBA</th>
<th>BRB</th>
<th>MVD(^a) Mean ± SE</th>
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<tr>
<td>–</td>
<td>–</td>
<td>5.7 ± 0.51</td>
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<tr>
<td>+</td>
<td>–</td>
<td>53.7 ± 5.6</td>
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<tr>
<td>+</td>
<td>+</td>
<td>22.6 ± 2.6(^b)</td>
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NMBA, N-Nitrosomethylbenzylamine; BRB, freeze-dried black raspberry powder; MVD, microvessel density.

\(^a\)MVD is expressed as number of microvessel per centimeter.

\(^b\)Significantly lower than rats treated with NMBA and fed control diets \((P < 0.0001)\).
acquisition of adequate oxygen and nutrients through the blood supply (34). Several cytokines and growth factors known to promote angiogenesis include transformation growth factor-β, transformation growth factor-α, platelet-derived growth factor, basic fibroblast growth factor and VEGF. VEGF is the most potent mitogen for vascular endothelial cells. Overexpression of VEGF has been strongly associated with angiogenesis in many human cancers including ESCC.

Our laboratory and research collaborators have been investigating the effects of freeze-dried BRB powder and BRB extracts in vivo and in vitro for the past 12 years. Black raspberries contain many known compounds with antioxidant and anti-inflammatory activities, such as flavonoid compounds, vitamins and phytosterols. However, the nature of the chemical constituents has not been fully delineated. In our previous studies, we demonstrated that BRB inhibits tumor development in NMBA-induced rat ESCC, azoxymethane-induced rat colon adenocarcinoma and 7,12-dimethylbenz(a)anthracene-induced hamster oral cavity cancer (30,35,36). Inhibition of tumor development by BRB may occur via the suppression of DNA adduct formation; inhibition of cell proliferation; or downregulation of COX-2, iNOS and transcription factor c-Jun (31). Biodirected fractionation studies have been conducted to identify the most active inhibitory components. BRB extracts and anthocyanins in BRB have been tested for their effects on gene expression in JB6 Cl 41 mouse epidermal cells (37,38) and rat esophageal epithelial cell lines (data not published). A methanol extract of BRB was found to inhibit benzo(a)pyrene-7,8-diol-9,10-epoxide-induced trans-activation of activator protein-1 and nuclear factor κB (NFκB) activity in JB6 Cl 41 cells (37). This same extract was found to inhibit VEGF expression through

Fig. 3. Correlations between VEGF, COX-2 and iNOS in NMBA-treated rat esophagus (A) and NMBA plus BRB-treated rat esophagus (B).
downregulation of the PI-3K/Akt pathway (38). An ethanol-water extract of BRB, and the anthocyanins; cyanidin-3-O-glucoside, and cyanidin-3-O-rutinoside, were all found to inhibit the proliferation rate and stimulate apoptosis in rat esophageal epithelial cells (Stoner et al.).

Potential correlations between VEGF and COX-2 and between VEGF and iNOS expression have not previously been assessed in ESCC. In rats treated with NMBA alone, we found a correlation between VEGF and COX-2 expression but not VEGF and iNOS or COX-2 and iNOS expression. In rats treated with NMBA plus BRB, however, we found correlations among all three factors: VEGF and COX-2, VEGF and iNOS, and COX-2 and iNOS.

The regulation of VEGF expression is a complex process wherein numerous genes and pathways are involved including Ras, COX-2 and iNOS. The Ras mutation contributes to tumor angiogenesis by enhancing the production of VEGF (39). In our previous study in rats, we observed Ha-ras codon 12 G→A transition mutations in all papillomas induced by NMBA (40).

The precise mechanisms involved in the suppression of VEGF by BRB and the suppression associated with the inhibition of COX-2 and iNOS by BRB are not fully understood and are most likely multiple in nature. One possible explanation is that BRB inhibit VEGF expression through downregulation of COX-2 and iNOS. COX-2 is an inducible enzyme that catalyzes the formation of prostanoids including PGE_2 in response to stimuli such as growth factors, tumor promoters, hormones and cytokines (41). The major contributions of COX-2 in carcinogenesis include enhancement of cell proliferation, resistance to apoptosis and enhancement of angiogenesis (42–44). Overexpression of COX-2 was observed in various human cancers including ESCC. Numerous studies show that COX-2 derived PGE_2 stimulate interleukin-6 and induce the expression of VEGF (45). PGE_2 binding to the prostaglandin receptor 2 (EP_2) increase the level of cAMP also leading to stimulation of VEGF (28). Overexpression of COX-2 itself was shown to upregulate Bcl-2 expression that results in vascular endothelial cell survival (46). Selective COX-2 inhibitors, such as NS-398 and JTE-522, were reported to effectively block angiogenesis by inhibiting VEGF expression (44,47). A positive correlation between COX-2 and VEGF was reported in numerous human cancers including non-small cell lung cancer (24), colorectal cancer (48), head and neck squamous cell carcinoma (49), endometrial carcinoma (50), renal cell carcinoma (51) and ovarian cancer (52).

iNOS is induced by certain cytokines, microbial products and lipo polysaccharides to catalyze the formation of NO and citrulline from L-arginine (53–55). Increased NO production is associated with many disorders, including cancer, through its reaction with oxygen and superoxide to produce peroxynitrite, which results in DNA damage (56). Numerous studies showed that iNOS may indirectly promote tumor angiogenesis through the induction of COX-2 and endothelial cell growth via elevated NO levels (57,58). A positive correlation between iNOS and VEGF was reported in human cancers, such as colon, lung and gastric cancers (29,59,60).

We previously found that BRB significantly decreases the expression of COX-2 and iNOS as well as the level of their metabolites, PGE_2 and NO, in NMBA-treated rat esophagus (37). With regard to VEGF, the present study suggests that BRB downregulates VEGF through its inhibitory effect on COX-2 and iNOS. A possible explanation for this observation is that some upstream molecules that control the expression of COX-2 and iNOS, such as PI-3K/Akt and NFkB, are modulated by BRB (37,38). The phosphatidylinositol 3’-kinase signaling cascade plays a central role in regulating cell proliferation and survival by affecting the phosphorylation status of Akt, a downstream molecule of the cascade (61). pAkt is commonly used as the index for the activation of Akt. A recent investigation showed that BRB extracts inhibit benzo(a)pyrene diol-epoxide (BPDE)–induced VEGF transcription by targeting the PI-3K/Akt pathway in mouse epidemial JB-6 Cl 41 cells (38).

NFkB is a pivotal transcription factor mediating the expression of many early response genes involved in carcinogenesis, including COX-2 and iNOS, and its overexpression is downregulated in BPDE-treated JB-6 Cl 41 cells (37,62,63). NFkB has two major subunits, p50 and p65. In normal cells, NFkB is sequestered in the cytoplasm in an inactive form through its association with its inhibitory protein, IκB-α. NFkB is activated by various signals, including cytokines, mitogens, environmental and occupational particles, and bacterial products. Some reports suggest that the activation of NFkB is also controlled by the activation of Akt (64). Activation of NFkB results in a degradation of IκB-α by phosphorylation and translocation of NFkB to the nucleus (65). pp65 and pIkB-α both are commonly used as an index for the activation of NFkB.

Based on the above molecular events, we assessed the expression of pAkt, pp65 and pIkB-α by immunohistochemistry in normal and NMBA-treated rat esophagus. In normal rat esophagus, positive staining of pAkt and pIkB-α was only observed in macrophages, not in epithelial cells; positive staining of pp65 was not detected in normal esophagus. In contrast, in esophagus treated with NMBA, we observed extensive cytoplasmic staining of pAkt and pIkB-α and nuclear staining of pp65 in the epithelium (unpublished data). In in vitro studies, our collaborators found that BRB extracts inhibit the activation of Akt and NFkB as indicated above. Thus, BRB may inhibit VEGF, COX-2 and iNOS through the suppression of upstream mediators such as Akt and NFkB.

In summary, we demonstrate for the first time that dietary BRB significantly inhibits VEGF-C expression and microvessel formation in NMBA-treated rat esophagus. Since angiogenesis plays a critical role in cancer progression, BRB may have anti-angiogenesis potential in cancer therapy. Moreover, we showed that the expression levels of VEGF, COX-2 and iNOS are correlated with each other in the esophagus of rats treated with NMBA and dietary BRB. Although the precise mechanisms remain to be elucidated, the modulation of angiogenesis by BRB appears to be associated with changes in the expression of COX-2 and iNOS. Thus, BRB may offer an advantage in cancer prevention and therapy by targeting multiple signaling pathways.

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References

22. Ratnasighe,D., Tangrea,J., Roth,M.J., Dowsey,S., Hu,N., Anver,M.,


26. Calviello,G., Nicuolo,F., Gragnolini,S., Piccioni,E., Serini,S., Maggiano,N.,


29. Kajita,T., Ohya,Y., Kimura,K., Tamura,M., Tanaka,Y., Tsumeuzaka,Y.,

30. Kresty,L.K., Morse,M.A., Morgan,C., Carlton,P.S, Lu,J., Gupta,A.,


35. Tsuji,M., Kawano,S., Sawaoka,H., Takei,Y., Kobayashi,I., Nagano,K.,


38. Huang,C., Huang,Y., Li,J., Hu,W., Aziz,R., Tang,M.S., Sun,N., Cassady,J.,

39. Aoki,K., Iwai,Y., Yamaguchi,M., Tomita,T., Terada,M., Moriyama,T.,

40. Liston,B.W., Gupta,A., Nines,R., Carlton,P.S., Kresty,L.A., Harris,G.K.,
