Contrasting roles of dietary selenium and selenoproteins in chemically induced hepatocarcinogenesis

Marina V. Kasaikina, Anton A. Turanov, Andrei Avanesov, Ulrich Schweizer, Sandra Seeher, Roderick T. Bronson, Sergey N. Novoselov, Bradley A. Carlson, Dolph L. Hatfield and Vadim N. Gladyshev

Division of Genetics, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115, USA, 1Institut für Experimentelle Endokrinologie, Charité-Universitätsmedizin Berlin, 13353 Berlin, Germany, 2Rodent Histopathology Laboratory, Harvard Medical School, Boston, MA 02115, USA, 3Department of Biochemistry, University of Nebraska, Lincoln, NE 68588, USA and 4Molecular Biology of Selenium Section, Laboratory of Cancer Prevention, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

*To whom correspondence should be addressed. Tel: +617-525-5122; Fax: +617-525-5147; Email: vgladyshev@rics.bwh.harvard.edu

Selenium (Se) has long been known for its cancer prevention properties, but the molecular basis remains unclear. The principal questions in assessing the effect of dietary Se in cancer are whether selenoproteins, small molecule selenocompounds, or both, are involved, and under which conditions and genotypes Se may be protective. In this study, we examined diethylnitrosamine-induced hepatocarcinogenesis in mice lacking a subset of selenoproteins due to expression of a mutant selenocysteine tRNA gene (TrspA37G mice). To uncouple the effects of selenocompounds and selenoproteins, these animals were examined at several levels of dietary Se. Our analysis revealed that tumorigenesis in TrspA37G mice maintained on the adequate Se diet was increased. However, in the control, wild-type mice, both Se deficiency and high Se levels protected against tumorigenesis. We further found that the Se-deficient diet induced severe neurological phenotypes in TrspA37G mice. Surprisingly, a similar phenotype could be induced in these mice at high dietary Se intake. Overall, our results show a complex role of Se in chemically induced hepatocarcinogenesis, which involves interaction among selenoproteins, selenocompounds and toxins, and depends on genotype and background of the animals.

Introduction

Many previous studies reported the antitumorigenic activity of dietary selenium (Se) (1–3). The Nutritional Prevention of Cancer clinical trial demonstrated that increasing dietary Se intake by selenized yeast led to a significant decrease in relative risk, number of cancer-related deaths and incidences of prostate, lung and colorectal cancers (4). Several other clinical trials have found that Se was effective in the early stages of carcinogenesis and that its chemopreventive activity strongly correlated with the initial Se status of human subjects (5–7). However, in the more recent SELECT (Selenium and Vitamin E Cancer Prevention Trial) study, selenomethionine supplementation was not effective in reducing prostate cancer incidence (5–7). This trial was terminated early due to the finding that neither Se nor vitamin E protected against prostate cancer. Moreover, higher vitamin E levels were associated with increased cancer risk and higher Se status with increased (but statistically not significant) risk of type II diabetes (8). Conflicting results have refueled the question whether the form of dietary Se is critical in cancer prevention and whether small molecule Se compounds or selenoproteins mediate the observed beneficial effects.

Epidemiologic studies have described associations of single nucleotide polymorphisms in selenoprotein genes (GPxs, SelP, Sep15, etc.) with cancer development, thereby arguing for mechanisms involving selenoproteins (7). Studies on several selenoproteins, e.g. thioredoxin reductase 1 (TR1 or TxnRd1) (9,10) and the 15 kDa selenoprotein (Sep15) (11), revealed contradictory functions of these selenoproteins in models of carcinogenesis. Although TR1 expression is often increased in cancer cells suggesting that this selenoenzyme may play a role in promoting cancer (see ref. 3 and references therein), its deficiency blocks self-sufficiency of growth and activation of checkpoints, and leads to altered DNA replication through a decreased expression of DNA polymerase alpha and reduction in the growth of xenografts (9,10). Contrasting observations, with regard to cancer, were made for Sep15 in a colon cancer model (11). In a model of chemically induced hepatocarcinogenesis using liver-specific TR1 knockout mice, a significant increase in liver tumors was observed (12) that further complicated these contrasting roles of TR1 in having a split personality in both preventing and promoting cancer. The loss of TR1 in tumors of these mice was compensated by overexpression of glutathione peroxidase 2 (GPx2) and upregulation of the glutathione system, suggesting the importance of redox control for tumor maintenance and growth (12).

Selenoproteins are proteins containing selenocysteine (Sec). Co-translational incorporation of Sec into proteins depends on tRNA^{Sec} (gene symbol Trsp). Decreased dietary intake of Se differentially impacts on the expression of selenoproteins. A hierarchy in selenoprotein expression protects the synthesis of housekeeping genes, such as TR1, although the expression of stress-related selenoproteins, such as GPx1, is drastically decreased upon Se deficiency (13). Thus, during Se deficiency, the balance among selenoproteins changes and may support neoplastic transformation by preserving selenoproteins essential for tumor progression, while decreasing the expression of antioxidant enzymes, such as GPx1. Deficiency in the stress-related selenoprotein population can also be induced by introducing a mutant Sec tRNA^{Sec} transgene (Trsp<sup>Arg<sup>[37G]</sup></sup>), wherein A at position 37 is changed to G and the resulting tRNA^{Sec}<sup>Arg<sup>[37G]</sup></sup> lacks the N<sup>6</sup>-isopentenyladenosine modification at position 37; and when the mutant transgene is expressed in high levels, a dramatic reduction in stress-related selenoprotein expression occurs (14). Thus, reduction in this selenoprotein subclass can be induced by a Se-deficient diet or by genetic manipulation of Trsp.

To distinguish the effects of selenocompounds and selenoproteins in Se chemoprevention, we compared tumor development in the mouse model of diethylnitrosamine (DEN)-induced hepatocarcinogenesis. We varied dietary Se status and independently interfered with selenoprotein expression by genetic modification of Trsp as described above. This approach allowed us to uncouple selenoprotein expression by both dietary and genetic manipulation from the effect of dietary Se. Our results indicate that hepatic selenoproteins have contrasting roles in the development of liver tumors. We found that the overall effect of dietary Se depended on experimental conditions and involved an interplay between selenoprotein status and levels of selenocompounds.

Materials and methods

Animals and treatment protocols

Se diets were based on a Torula yeast Se-deficient diet that contained 0.01 p.p.m. Se. They were purchased from Harland TekLad (Madison, WI).
The Se-deficient diet was supplemented with 0 (Se deficient), 0.1 p.p.m. (adequate), 0.4 p.p.m. (Se supplemented) or 2.25 p.p.m. (high Se) of sodium selenite, as described previously (15). Se was included in the diets in the form of sodium selenite. The following rationale was used for selecting the diets: 0.1 p.p.m. Se was a minimal amount of Se that is sufficient for nearly maximal expression of stress-related selenoproteins. Because maximal expression of GPx3 is achieved at 55 μg/day Se in humans, the 0.1 p.p.m. Se mouse diet may be viewed as approximately corresponding to the Recommended Dietary Allowance for adult humans. Likewise, the human diet with 200 μg Se/day, which is the amount of Se that has been extensively used in clinical trials, would correspond to the 0.4 p.p.m. Se mouse diet. In addition, we employed the 2.25 p.p.m. Se diet because it represents the amount of Se required for the chemopreventive effect of Se in rodent models. It should be noted, however, that the mouse diets used in this study can only partially be extrapolated to humans (e.g. severe Se deficiency and high Se conditions very rarely occur in humans).

Mice overexpressing a mutated tRNA[Ser]Sec allele, TrspA37G, in a Friend Virus B/N genetic background have been described previously (16). These mice lack the N4-isopentenyladenosine modification of A37 in tRNA[Ser]Sec, TrspA37G and control female mice (8–12 mice per group on FVB/N genetic background) were placed on the indicated Se diets at day 1 after birth by providing Se diets to the dams. Fourteen days later, pups received a single intraperitoneal injection of DEN (10 μg/g body weight). At 30 days, mice were weaned and continued on the same Se diets for an additional 5.5 months. At the endpoint of the experiment, animals were sacrificed and their tissues were collected for further analyses.

75Se labeling and immunoprecipitation of selenoproteins

Experimental and control (FVB) mice (6 month old) were injected with 40 μCi of freshly neutralized 75Se-selenious acid and maintained on a 12h light/dark cycle for 48h. Then, mice were killed and protein extracts prepared from various tissues in ice-cold phosphate-buffered saline, pH 7.6, supplemented with complete protease inhibitor mixture (Roche Applied Science). These extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by PhosphorImager analysis (left panel) and immunoprecipitation with antibodies specific for indicated selenoproteins (right panel). (A) FVB (control) and TrspA37G mice were metabolically labeled with 75Se by intraperitoneal injection, and 48 h later sacrificed and their tissues collected and labeled selenoproteins analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by PhosphorImager analysis (left panel) and immunoprecipitation with antibodies specific for indicated selenoproteins (right panel). (B) Experimental design and timing of DEN injection and tumor analysis.

Pathology analyses

Liver and brain tissues were dissected and immediately fixed in 10% neutral buffered formalin and 24–48h later washed with phosphate-buffered saline. Further tissue embedding and hematoxylin/eosin staining were performed in the Dana-Farber rodent histopathology core. For immunohistochemistry, 5 μm sections were deparaffinized in xylene and stained with Ki67 antibodies with Histostain Plus Kit (Zymed) according to manufacturer’s instructions. Semiquantitative analysis was carried out using ImageQuant software. Brain sections were cut on a vibratome at 25 μm and stained for glial fibrillary acidic protein (GFAP) (Dako, Denmark) and parvalbumin (Swant, Bellinzona, Switzerland) as described (17).

Western blot analysis

Tissue extracts were prepared in ice-cold phosphate-buffered saline, supplemented with complete protease inhibitor mixture (Roche Applied Science). Expression of selenoproteins GPx1, TR1, TR3 and MsrB1 was analyzed by western blots with polyclonal antibodies specific for these proteins.

Results

Mouse models to assess the roles of Se and selenoproteins in hepatocarcinogenesis

Mouse selenoproteins can be divided into ‘housekeeping’ and ‘stress-related’ selenoproteins according to their response to Se deficiency (18). Under conditions of reduced dietary Se, the levels of stress-related selenoproteins, such as GPx1, MsrB1 and SelW, significantly decrease, whereas the levels of housekeeping selenoproteins, such as TR1 (TxnRd1) and TR3 (TxnRd2), decrease only slightly. Reduction in dietary Se is mostly associated with the decreased expression of stress-related selenoproteins, often due to the effects on both translation and selenoprotein mRNA abundance. To investigate whether stress-related selenoprotein deficiency is associated with liver cancer development, we used TrspA37G mice (18). As noted in the Introduction, overexpression of the TrspA37G transgene impairs biosynthesis of stress-related selenoproteins.

To assess the selenoprotein status of TrspA37G mice, wild-type (FVB) and TrspA37G mice were metabolically labeled with 75Se for 48h. PhosphorImager analysis showed a clear difference in selenoprotein
expression between these mice, i.e. expression of stress-related selenoproteins (e.g. GPx1) was selectively decreased in the transgenic mice compared with wild-type (control) mice (Figure 1A, left panel). Immunoprecipitation of several selenoproteins further highlighted the selective impairment of selenoprotein biosynthesis. The levels of housekeeping selenoprotein expression (e.g. TR1 and TR3) were less affected compared with significantly decreased expression of stress-related selenoproteins (SelS and MsrB1) in the Trsp(A37G) mice. Thus, Trsp(A37G) decreased selenoprotein expression (compared with control FVB/N mice) under conditions of sufficient dietary Se (Figure 1A, right panel).

To evaluate the role of stress-related selenoproteins in hepatocarcinogenesis, we chose the DEN model of chemically induced carcinogenesis. In addition, to compare the effects of genetic and dietary selenoprotein deficiency, each mouse model was placed on four Se diets: a Se-deficient diet and the same diet supplemented with 0.1 p.p.m. (adequate), 0.4 p.p.m. (Se supplemented) and 2.25 p.p.m. (high Se) of sodium selenite from birth (Figure 1B).

High mortality of Trsp(A37G) mice on both Se-deficient and Se-enriched diets

Although the study was aimed at studying tumorigenesis, we first observed a striking phenotype associated with Se status. Starting at day 60 on the Se-deficient diet, DEN-injected Trsp(A37G) mice began developing a walking behavior, running in one direction in a circular pattern with frequent loss of balance, similar to Trsp(ΔStaf) mice described previously (14). Shortly after, these mice developed lower limb paralysis and died within several days (Figure 2). In contrast, none of the corresponding mice on the Se-supplemented diet showed this phenotype during the experiment. Surprisingly, after ~100 days, Trsp(A37G) mice on the high Se diet also began developing neurologic phenotypes. However, kinetics of disease onset and development were different (Figure 2). Although 80% of Trsp(A37G) mice on the Se-deficient diet showed this phenotype and had to be sacrificed at different time points during the experiment starting at day 60, Trsp(A37G) mice on the high Se diet developed the neurological phenotype later (starting day 100) and all of them either died or had to be sacrificed within 14 days from the onset (Figure 2).

In order to better understand the neurological phenotypes, we studied brain histology in Trsp(A37G) mice. Nissl staining did not reveal widespread cell death in the cortex or a thinning of hippocampal neuronal layers. Loss of parvalbumin neurons has been associated with selenoprotein deficiency (14,17,19). We did not observe a loss of parvalbumin-positive neurons in the barrel field-cortex in Trsp(A37G) mice fed either low Se or high Se diet as compared with adequate diet, on which no neurological phenotype was observed (data not shown). Another hallmark feature of selenoprotein deficiency in the brain is massive astrogliosis that is seen as increased numbers of GFAP-positive cells in the cerebral cortex of Trsp(ΔStaf) mice and mice lacking neuronal selenoproteins. To our surprise, no astrogliosis was detected in the cortices of Trsp(A37G) mice and even around blood vessels and close to white substance, GFAP appeared reduced in the mutants irrespective of Se content in the diets (Figure 3A). Moreover, in the hippocampal formation, a region with constitutively high GFAP expression in normal mice, GFAP was notably reduced in transgenic mice (Figure 3B). These findings show a novel role of stress-related selenoproteins in the expression of GFAP in astrocytes, however further studies are required to provide the explanation of the observed Se-dependent neurological symptoms of TrspA(37)G mice.

Deficiency in stress-related selenoproteins promotes tumor development

Six months after DEN injection, mice were sacrificed and their livers analyzed for occurrence of tumors. Because Trsp(A37G) mice on both Se-deficient and high Se diets were largely lost due to neurological phenotypes, we first focused on comparison of FVB/N control and

---

**Fig. 2.** Survival of Trsp(A37G) mice maintained on Se diets. FVB (wild-type) and Trsp(A37G) mice were maintained on indicated Se diets and injected with DEN. The graphs show survival of animals during the course of the experiment.
M.V. Kasaikina et al.

Trsp\textsuperscript{A37G} mice consuming the 0.4 p.p.m. Se diet. This analysis revealed higher tumor incidence in transgenic mice (Figure 4A). Trsp\textsuperscript{A37G} mice had an incidence of carcinoma, which was not seen in wild-type mice (Figure 4B); they also featured larger tumors that had much higher Ki67-containing cells (Figures 4C and D). This is reminiscent of similar findings in a transgenic mouse model of prostate neoplasia (20). In

Fig. 3. Expression of GFAP is significantly reduced in the brains of Trsp\textsuperscript{A37G} transgenic mice. (A) GFAP expression in somatosensory cortex. Astrocytes in white matter, along the meninges, and around blood vessels are normally expressing GFAP. In Trsp\textsuperscript{A37G} mice, including those fed with low Se and high Se diets, GFAP expression is reduced, although astrogliosis and increased GFAP are normally associated with neurodegenerative disease. (B) In the hippocampal formation, high GFAP expression is normal. In Trsp\textsuperscript{A37G} mice, GFAP expression is clearly reduced irrespective of Se content in the diet.

Fig. 4. Stress-related selenoproteins and liver tumor development. Mice were maintained on the 0.4 p.p.m. Se diet and subjected to DEN injection. (A) Incidence of liver lesions, including abscesses, necrosis, adenomas, carcinomas and fatty nodules in FVB and Trsp\textsuperscript{A37G} mice. Numbers of tumor-bearing animals and a total number of animals in the group (in parentheses) are shown above the bars. (B) Representative images of livers dissected from FVB and Trsp\textsuperscript{A37G} mice. Visible liver lesions are indicated with arrows. (C) Representative images of liver sections from tumor and surrounding normal tissues, stained with Ki67 antibodies. Ki67-containing cells are visualized by the more darkly stained cells. (D) Quantification of Ki67 immunostaining. Four animals per group were analyzed and three fields of view per section were averaged. (E) Western blot analysis of selenoproteins in livers and kidneys of FVB and Trsp\textsuperscript{A37G} mice. Samples in different lanes represent different animals.
addition, TrspA37G mice exhibited signs of inflammation that resulted in necrosis and abscesses (Figure 4A). The development of different liver lesions (besides tumors) might provide insights into the contributions of selenoproteins to various processes, e.g. necrotic cell death. Thus, deficiency in stress-related selenoproteins under conditions of sufficient dietary Se was associated with elevated tumorigenesis.

To verify influence of the TrspA37G transgene on selenoprotein status in these DEN-injected mice, we analyzed selenoprotein expression in liver and kidney. Western blotting revealed that GPx1 and MsrB1 were essentially missing under conditions of the Se-deficient diet, whereas the expression of housekeeping selenoproteins (TR1 and TR3) was altered much less significantly (Figure 4E).

Both Se-deficient and Se-enriched diets protect FVB mice from tumor development

In order to assess the influence of dietary Se on DEN-induced tumorigenesis, we further compared the development of liver tumors in FVB/N mice maintained on Se-deficient, Se-adequate, Se-supplemented and high Se diets (Figure 5A). Interestingly, mice on both Se-deficient and highly enriched Se diets were fully protected. This finding was also consistent with histological analyses of liver sections and Ki67 staining (Figure 5B and C). Thus, selenoprotein deficiency associated with low dietary Se, in contrast with the effect of the TrspA37G transgene, protected against tumor development. However, because high levels of Se were also protective, the data suggest that it is the response to toxicity associated with very high (i.e. selenite itself may be toxic due to generation of reactive oxygen species) or very low (i.e. compromised selenoprotein function leads to elevation of reactive oxygen species and other toxic compounds) dietary Se that is associated with the protection.

We again used western blotting to assess selenoprotein status in these mice. As expected, they lacked GPx1 expression under Se-deficient conditions, whereas GPx1 levels were increased with the increase in dietary Se (Figure 5D). Suppression of tumor development in the chemical carcinogenesis model is consistent with the previous findings in c-myc/TGFα transgenic mice, where both Se-deficient and Se-enriched diets protected against hepatocarcinogenesis (15).

Regulation of stress-related protein expression by selenoprotein and Se status

To obtain insights into the contrasting roles of selenoprotein status in chemically induced tumor development, we assessed the expression of marker genes in TrspA37G and FVB mice subjected to DEN injection. For comparison, we examined mice not treated with DEN as well as mice in another background, C57BL6/129, which are resistant to DEN-induced carcinogenesis (Figure 6). Some of the samples represented mice that could not be analyzed for cancer incidence due to high mortality among the TrspA37G animals on the 2.25 p.p.m. Se diet, but their remaining samples could be examined by western blotting. TR1 expression could be somewhat reduced in TrspA37G mice independent of dietary Se status, and the expression of this protein was also decreased by Se deficiency in both FVB and C57BL6/129 backgrounds (Figure 6A). Because both Se deficiency and high levels of this micronutrient are known to increase glutathione S-transferase GSTA1 levels, we assessed expression of this protein in the samples. GSTA1 was induced by high dietary Se in both TrspA37G and FVB mice. However, its expression was elevated by Se deficiency more significantly in FVB than in C57BL6/129 mice (Figure 6A).

Consistent with the fact that elevated expression of GSTA1 depends on the NRF2 pathway, expression of another NRF2-dependent gene,
The aim of this study was to assess the role of dietary Se and selenoproteins in cancer prevention in a mouse model of chemically induced hepatocarcinogenesis. To address this question, we induced liver tumors in transgenic mice lacking a subset of selenoproteins, in which selenoprotein expression was decreased independent of dietary Se status, thereby allowing us to assess the roles of groups of selenoproteins under Se normal dietary conditions as well as under conditions of Se deficiency and excess. This model, TrspA37G mice, overexpressed a mutant form of Sec tRNA[Ser]^{Sec}, leading to reduced expression of stress-related selenoproteins, whereas housekeeping selenoprotein expression was only slightly affected. These mice represent a convenient model as the expression of multiple non-essential, stress-related selenoproteins is affected (e.g. GPx3, GPx1 and SelW), although low levels of expression of some selenoproteins preclude assessment of their expression levels. We reasoned that subjecting these animals and the corresponding control animals of the same background to Se diets would enable us to distinguish the effects of selenoproteins and low molecular weight Se compounds on hepatocellular carcinogenesis. However, our data revealed a highly complicated picture of

**Discussion**

The findings with FVB mice were consistent with the previous results observed in our laboratories with c-myc/TGFα mice as well as with other published research (15,21), and may potentially be explained by the induction of the NRF2 pathway by oxidative stress, resulting from both selenoprotein deficiency and excess of dietary Se (22). Induction of the NRF2 pathway in response to selenium deficiency was studied in several models. Deletion of the Trsp gene in macrophages resulted in an increased expression of NRF2 target genes, including glutathione S-transferase P1 and NAD(P)H:quinone oxidoreductase 1. Simultaneous ablation of the NRF2 and Trsp led to poor viability and increased susceptibility to hydrogen peroxide treatment (23). In general, the NRF2 pathway could mobilize antioxidant and other protective enzymes, protecting against malignant transformation (24). This idea is also supported by the analysis of expression of NRF2-dependent genes. We also observed differences in the expression of NRF2-dependent genes depending on background of mice, e.g. comparing more susceptible FVB mice with C57BL6/129 mice, which are more resistant to carcinogenesis. Thus, genotype, including both transgene expression and background of mice, influenced the outcome of DEN injection.

In the course of our study, we made further interesting observations: TrspA37G mice, maintained on the Se-deficient diet developed a neurologic phenotype similar to that in mice expressing a hypomorphic tRNA[Ser]^{Sec} allele (14). Interestingly, the reduction of parvalbumin expressing interneurons seen in other mouse models seems independent from stress-related selenoproteins because TrspA37G mice express normal levels of housekeeping interneurons and retain normal numbers of interneurons. This finding is in line with our earlier proposal that parvalbumin-positive interneurons specifically depend on GPx4, a housekeeping selenoprotein (17,19). Interestingly, this means that reduced expression of stress-related selenoproteins, at least if all are affected simultaneously, is sufficient to induce a neurological phenotype implicating one or more stress-related selenoproteins in neuronal function. Loss of deiodinase 2 may contribute to developmental defects in the inner ear (25), but this has not been investigated in our mice. A surprising finding was the apparent dependence of astrocytic GFAP expression on stress-related selenoproteins. SelS was previously found to be induced in astrocytes during cellular stress (26). Future studies will be needed to address the role of this and other selenoproteins in astrocyte function. Because none of the TrspA37G mice fed adequate or supplemented Se diets showed the neurological phenotype, we suspect that the effect is related to expression levels of selenoproteins and not to any effects of transgene integration. At present, it is hard to explain why feeding TrspA37G mice a high Se diet also induced a neurological phenotype, albeit with different kinetics.

Overall, when viewed together with previous analysis of the role of Se and selenoproteins in cancer, our study reveals a complex role of dietary Se in cancer chemoprevention, which is not limited to the role of selenoproteins in cellular pathways leading to malignant transformation. A combination of toxicity of carcinogens, dietary Se and selenoprotein status appears to underlie the effects of Se, with further
contributions from genotype. If these findings can be transferred to the human situation, they would expose complexities that could not be addressed in clinical trials. Therefore, further investigations of model organisms to define the conditions under which Se and/or selenoproteins decrease cancer incidence are required to further elucidate these complexities.

Funding

National Institute of Health (CA080946 to V.N.G.); Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National Institute of Health (to D.L.H.).

Conflict of Interest Statement: None declared.

References

15. Novoselov, S.V. et al. (2005) Selenoprotein deficiency and high levels of selenium compounds can effectively inhibit hepatocarcinogenesis in transgenic mice. Oncogene, 24, 8003–8011.

Received October 4, 2012; revised December 31, 2012; accepted January 8, 2013