Research Article

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Glutathione S-Transferase-π Levels of In Vitro Generated Epithelial Mesenchymal Transition Model in Caco-2 Cell Line

Caco-2 Hücre Hattı *In Vitro* Epitelyal Mezenkimal Geçiş Modelinde Glutatyon S-Transferaz-π Düzeyleri

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ABSTRACT

Despite the developments in the modern medicine, the resistancy to adjuvant/neoadjuvant therapies is one of the most important problem that may complicate the treatment. In recent years, studies have shown that activation of epithelial-mesenchymal transition (EMT) may plays an important role in cancer cells tendency to metastasis and resistance to chemotherapy. In this work, in vitro EMT model was generated with Caco-2 cell line to determine the mechanisms of response and resistancy. This cells are well-known for their ability to transform and are important tools for structural and functional studies due to their differentiation potential. Differentiation was monitored on days 0th, 15th and 30th and epithelial and mesenchymal markers (E-cadherin and Vimentin) were used for model validation with qRT-PCR. Glutathione S-Transferase-π (GST-π) levels were determined to elucidate the mechanisms of resistancy in both forms of EMT with ELISA. As expected, it was shown that GST-π levels increased significantly in mesenchymal form. Also, GST-π inhibitor hypericin was used to eliminate resistancy in mesenchymal form. Significant decrease in GST-π levels was determined with the different concentrations of hypericin treatment (2μM and 20μM). These results showed that, activation of mechanisms to eliminate resistancy in addition to adjuvant therapy may increase the effectiveness of treatment.

Keywords: Colorectal cancer, Epithelial Mesenchymal Transition, Glutathione S-Transferase-π

ÖZET

Modern tıptaki gelişmelere rağmen, adjuvant/neoadjuvan tedavilere direnç gelişimi tedaviyi karmaşıklaştırabilecek en önemli sorunlardan biridir. Son yıllarda, kanser hücrelerinin epitelyal mezenkimal geçiş (EMT) aktivasyonunun, metastaz eğilimi ve kemoterapiye direncinde önemli bir rol oynadığını göstermiştir. Bu çalışmada CaCo-2 hücre hattında in vitro EMT modeli oluşturularak direnç mekanizmalarının belirlenmesi hedeflenmiştir. Bu hücreler dönüşüm yetenekleri açısından iyi bilinen hücre hatlarıdır ve farklılaşma potansiyelleri nedeniyle yapısal ve fonksiyonel çalışmalar için önemli araçlardır. Farklılaşma 0., 15. ve 30. günlerde takip edilmiştir ve model validasyonu için epitelyal ve mezenkimal belirteçler (E-kadherin ve Vimentin) kullanılmıştır. EMT'nin iki formunda da direnç

mekanizmalarını aydınlatmak üzere ELISA ile Glutatyon-S-Transferaz-π (GST-π) düzeyleri analiz edilmiştir. Mezenkimal formda beklendiği şekilde GST-π düzeyinin önemli derecede arttığı gösterilmiştir. Ayrıca mezenkimal formda direnci ortadan kaldırmaya yönelik GST-π inhibitörü hiperisin kullanılmıştır. Hiperisinin farklı konsantrasyonları (2μM ve 20μM) ile muamele sonucu GST-π düzeylerinde anlamlı derecede azalma tespit edilmiştir. Bu sonuçlar, adjuvan terapiye ek olarak direncin ortadan kaldırılmasını sağlayacak mekanizmaların aktifleştirilmesi ile kullanılan tedavinin etkinliğinin arttırabileceğini ortaya koymuştur.

Anahtar Kelimeler: Kolorektal kanser, Epitelyal Mezenkimal Geçiş, Glutatyon S-Transferaz-π

1. Introduction

Epithelial-Mesenchymal Transition (EMT) is an embryonic differentiation that facilitates the formation of highly mobile cells with stem cell properties (1). Most cells originating from the ectoderm or mesoderm contain EMT factors, and their mesenchymal properties constitute their differentiation markers (2). Only cells that originate from the endoderm show EMT transition at certain stages of development and turn into differentiated cells (3). Differentiation is common in cancer progression. Mostly, mesenchymal markers are acquired with the loss of epithelial features and transition to metastasis develops (4, 5).

Conversion of an epithelial cell to mesenchymal cell shows changes in morphology and migration properties. Common molecular markers for EMT are: E-cadherin, vimentin, transgelin, fibronectin, caludin4 (6-8). Markers for EMT are increased with the acquired migration capacity and apoptosis resistancy (9, 10). The opposite is valid for MET (mesenchymal epithelial transition). Cancer cells begin to show stem cell properties and resistance to chemotherapeutic drugs (11). In addition to phenotypic differences between epithelial form and mesenchymal form, significant differences were found in terms of resistance to chemotherapeutic drugs (12, 13). Tumor cells in mesenchymal form exhibit a more resistant profile to drugs. Epithelial cells transformed into EMT acquire a more mobile and invasive phenotype and become more resistant to anticancer drugs (13, 14). It has been suggested that mesenchymal-epidermal transition plays a role in tumor colonization in metastatic regions (14). And caco-2 cell lines are well known for their ability to convert into mature intestinal cells. Due to their differentiation potential, these have become important for studies of EMT in vitro [18].

EMT-induced chemotherapy resistance has been identified in different cancer models; However, the cellular mechanism of the phenomenon is still unclear. Chemotherapy resistance is thought to have 3 causes. Cancer cells can alter the flow of chemotherapeutic agents, activate effective antioxidant systems such as Glutathione S transferases, provide effective DNA repair to avoid apoptosis from DNA damage. Recently, the role of oxidative stress in the development of colorectal cancer is well discussed (15). Cells is not affected with free radicals if oxidative stability is normal. The imbalance of formation of radicals may cause deterioration (15).

Glutathione S Transferases (E.C.2.5.1.18, GST) are members of a large family of enzymes that catalyze the conjugation of nucleophilic glutathione (GSH) to electrophilic xenobiotics and endobiotics (16, 17). Furthermore, they directly bind to various non-substrate molecules and mediate intracellular transport of these molecules (18). Recent studies have shown that GSTs also function in the synthesis and metabolism of prostaglandins, leukotrienes and steroids, in reducing the harmful effects of toxic products caused by oxidative stress and in signal transduction pathways (19). Studies have been conducted on GST inhibitors in order to increase the effectiveness of treatment (16-19). Hypericin is candidate molecule in the treatment of tumors (Figure 1) (20).

In this study, it is aimed that the establishment of *in vitro* EMT model of CaCo-2 cell line, the determination of levels of GST-pi in both forms and the elimination of resistance mechanism with hypericin.

2. Materials and Methods

2.1. Epithelial Mesenchymal Transition

Caco-2 cell line were cultured in Dulbelco's Modified Eagles Medium (DMEM) supplemented with 20 % fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.5 gL^{-1} sodium bicarbonate and 1 mM sodium pyruvate. They were allowed to grow in an environment containing 5 % CO2 at 37 °C. Under these conditions, the cells reached confluent within an average of 10 days. 95% confluent

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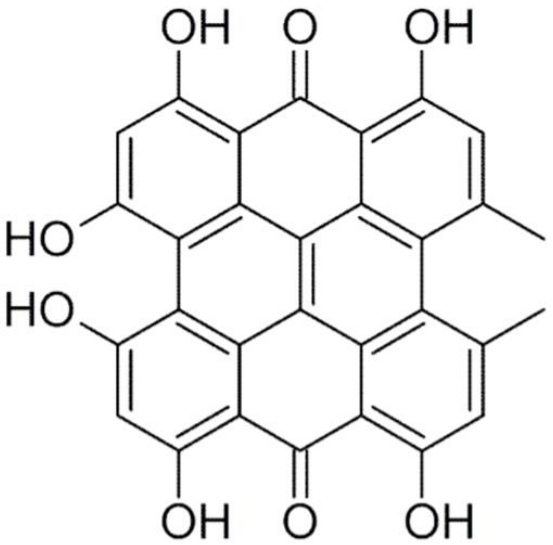

Figure 1. Chemical structure of hypericin.

cells were considered as day 0. for the follow-up of differentiation. The cells were then continued to grow in the same medium. Samples were collected at 0th 15th and 30th days. Time-dependent proliferation and transition of the cells were monitored. The medium were collected at various intervals, on days 0^{th}, 15^{th}, and 30^{th}. Differentiation was validated by Real-time polymerase chain reaction (RT-PCR) using EMT markers (for epithelial form; E-cadherin; for mesenchymal form; Vimentin). Parallel cultures were performed to provide replicated differentiation sets.

2.2. RNA Isolation

Cells were washed with 1X Phosphate buffered saline (PBS) and then scraped with 1X PBS. After centrifugation, the supernatant was removed and 1ml of TRIzol was added. The cells were homogenized by pipetting and incubated with TRIzol for 5 minutes at room temperature. Then 200µl of chloroform was added. The mixture was then vigorously stirred and incubated at room temperature for 10 minutes and centrifuged at 13,000 rpm 4 °C for 15 minutes. The supernatant was removed and 500µl of isopropanol was added. The mixture was gently inverted and incubated at room temperature for 10 minutes, then centrifuged at 13,000 rpm 4 °C for 15 minutes. The pellet was washed with 75% ethanol and the ethanol was removed by centrifugation. The pellet was air dried and then resuspended in desired nuclease-free water. The RNA was incubated at 55 °C for 15 minutes, then were stored at -80 °C for long storage.

2.3. Quantitative Real Time-Polymerase Chain Reaction

RNA samples were prepared by phenol-chloroform extraction method using TRIzol reagents. 500 ng of RNA was reverse transcribed by using random hexamer primers, RNAase inhibitor and Revert-Aid first strand cDNA synthesis kit. All PCR reactions were repeated 3 times using Sybr Green and gene expression mixtures. Using Q RT-PCR, an increase/decrease of the markers (E-cadherin / Vimentin) were determined in the epithelial and mesenchymal form of CaCo-2 EMT cell model. GAPDH is used as an endogenous control in the analysis of Q RT-PCR. The used primers are given (Table 1).

2.4. GST-π ELISA

The interaction between glutathione and 1-Chloro-2,4-dinitrobenzene (CDNB) is entirely dependent on the presence of active GST. The GST activity assay kit works based on the GST-catalyzed reaction between glutathione and GST substrate CDNB. GS-DNB generated by GST catalysis produces dinitrophenol thioether and this is detected by spectrophotometer at 340nm. 1 unit of GST activity is defined as the amount of enzyme producing 1µmol GS-DNB conjugate per 1 minute. The kit detects GST activity in cell lysate. In order to determine the activity

Table 1. Primer sequences used in RT-PCR experiments.

Primers	Sequences	
GAPDH	F:5'-ATGGGCAGCCGTTAGGAAA-3'	R:5'-GCATCGCCCCACTTGATTTT-3'
E-Cadherin	F:5'-TGGGCCAGGAAATCACATCCTACA-3'	R:5'-TTGGCAGTGTCTCTCCAAATCCGA-3'
Vimentin	F:5'-CCAAGACACTATTGGCCGCCTGC-3'	R:5'-GCAGAGAAATCCTGCTCTCCTCGC-3'

of GST-π isoenzyme, GST-π levels were determined separately on days 0th, 15th and 30th in Caco-2 cells with ELISA kit.

2.5. Cytotoxicicity

The mesenchymal form of Caco-2 cells (day 30) was treated with 2μM and 20μM Hypericin and then GST levels was determined by ELISA.

2.6. Data Analysis

Data analysis was performed using Excel, Graphpad softwares available in our laboratory.

3. Results and Discussion

Although there are various opinions about the drug resistance, one of the most important factor is thought to be the epithelial - mesenchymal transition of some tumor cells. In EMT, epithelial cells acquire invasive and migratory properties, and contributes to the formation of metastases during carcinogenesis. The reason why tumor cells in mesenchymal form are more resistant, invasive and have poor prognosis. The importance of EMT is well investigated in several studies (21, 22). Chen et al. demonstrated that EMT has important roles in differentiation of cancer cells (23).

Caco-2 cells have appropriate cell division and produce normal cells at low density. Studies have shown that the Caco-2 cell line appears to be ideal for EMT modelling [19-21]. To describe this model and to define transcriptional regulation in depth some studies were performed [18-21]. Halbleib et al. showed that many other proteins required for epithelial binding complexes (protocadherins and desmosomal cadherins) are regulated at the transcriptional level [20]. The study has demonstrated that transcriptional regulation has a vital role in the differentiation of Caco-2 cells into fully functional enterocytes without the necessary normal signals *in vivo* [20]. Saaf et al. showed that differentiated Caco-2 cells resemble normal epithelial cells more than tumor cells [21].

To investigate the critical roles of EMT, the transmission was established in CaCo-2 cell line in this study. As expected, epithelial and mesenchymal forms of CaCo-2 cells were actually different from each other by their morphological patern (Figure 2).

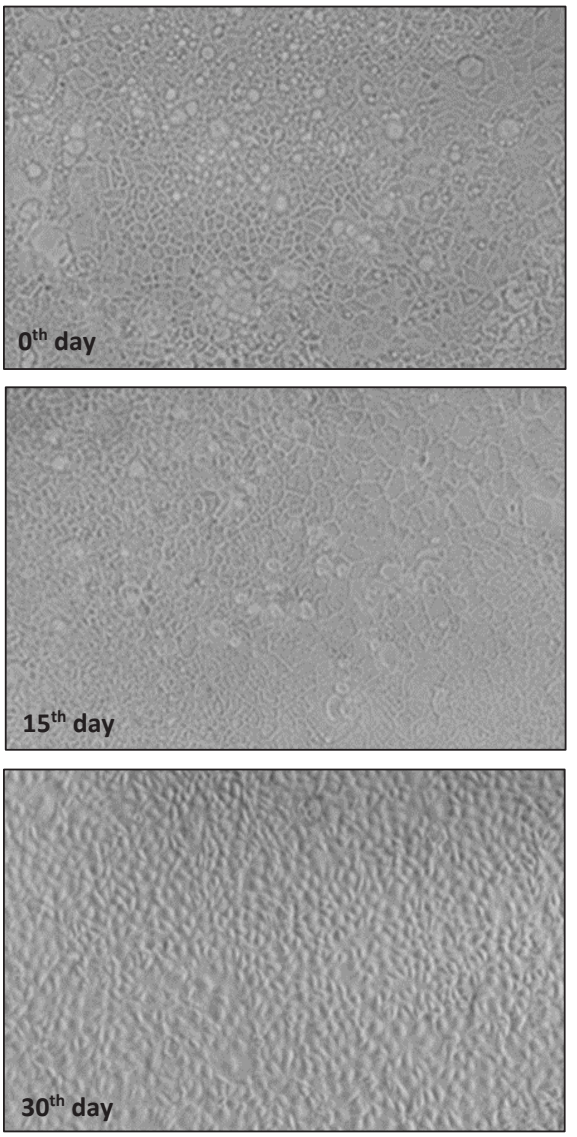

Figure 2. Time-dependent cell morphology of Caco-2 cell line on days 0th 15th and 30th.

Down regulation of e-cadherin is very important in epithelial-mesenchymal transformation. E-cadherin has been shown to be overexpressed in tumor cells in epithelial form, whereas expression in mesenchymal forms has been shown to decrease. While vimentin is overexpressed in mesenchymal cells; is less expressed in epithelial form [9]. The transmission gives cells the ability to regenerate.

After the model was established, it was important to validate the transmission with epithelial - mesenchymal markers (for epithelial form 'e-cadherin' and mesenchymal form 'vimentin'). According to RT-PCR results, E-cadherin levels decrased significantly

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at day 0th cells (epithelial form) compared with day 30th cells (mesenchymal form). As expected, mesenchymal marker vimentin was higher at day 30th cells when compared to day 0th cells. This indicates that EMT transition has been performed *in vitro* successfully (P <0.001) (Figure 3).

Mesenchymal cells show a highly invasive and highly motile phenotype. The mesenchymal form is therefore likely to be more resistant to ambient conditions, oxidative stress, and an increase in the expression of antioxidant enzymes produced in response to oxidative stress. However, there are evidences that cancers activate EMT programs to achieve undifferentiated status and gain resistance to treatments (24).

It is known that tumor cells are more resistant, aggressive and invasive in the mesenchymal form. It is highly probable that antioxidant system enzymes such as Glutathione S transferases have elevated roles (e.g. increased resistance to oxidative stress) in the mesencymal form. Increased resistancy or invasiveness could be related to increased oxidative stress elements and so associated to increased detoxification system enzymes such as Glutathione S transferases. So, it is hypothesized that cells may have increased Glutathione S transferase levels in the mesenchymal form and this could be one of the reason of resistancy. Consequently resistancy to antitumor drugs may develop. It is also known that Glutathione S transferase have functions in reducing harmful effects of toxic products caused by oxidative stress and in signal transduction pathways. Therefore, Glutathione S transferase levels in mesenchymal form were expected to increase while under cellular stress.

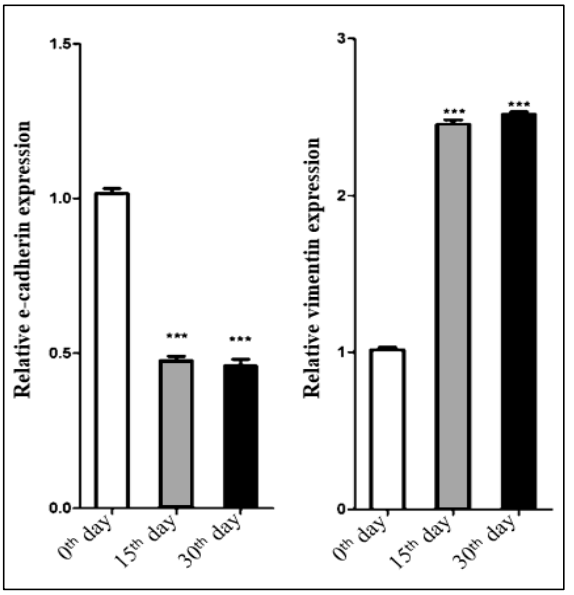

Figure 3. Gene expression of relative e-cadherin (A), Gene expression of relative vimentin (B) Endogenous control is GAPDH. (*** P <0.001 versus day 0th).

Our results demonstrated that as expected GST-π levels was significantly higher in mesenchymal form than epithelial form (p<0.01). These results supported that mesenchymal form is more resistant to oxidative stress than epithelial form (Figure 4). This shows resistancy mechanisms are activated in the mesencymal form. Similerly Mani et al. suggested that human mammary epithelial cells exhibiting EMT gain stem cell characteristics (13). Giannoni et al. reported that EMT can be promoted in carcinoma cells and allows the cell to acquire stem cell properties (25). Basu et al. have stimulated the *in vitro* model and have subjected the cell lines to hypoxic stress and showed aggressive behaviour of prostate cancer (26).

High levels of GST-π were found in cancer cell lines and chemotherapy resistant cell lines. There are studies indicating that these enzymes can be used as a sensitive biomarker for cancer development (16, 18). Hypericin is candidate molecule in the treatment of tumors (20). It has been shown to accumulate in the endoplasmic reticulum, golgi complex, plasma and nuclear membranes. Turk et al. showed that hypericin is bound to GST and decrease its catalytic activity (27). It is reported that there was a strong reduction in the catalytic activity of GST in cells, where hypericin tightly binds GST-π. GSTs are provide oxidant / antioxidant balance and its inhibition with hypericin suggests that the treatment efficacy may change by using some inhibitors. These findings reveal the importance of GST-hypericin interaction (27-30).

Thus, it is aimed to decreasing resistancy by using GST inhibitor in the mesencymal form. The inhibitor is expected to affect the resistance mechanism. After hypericin treatment, It was determined that hypericin treatment significantly decreased (P <0.05) GST-π levels without dose dependent (Figure 5). It is suggested that inhibitor treatment can reduce GST-π and indirectly reduce the resistance in the mesenchymal form as expected.

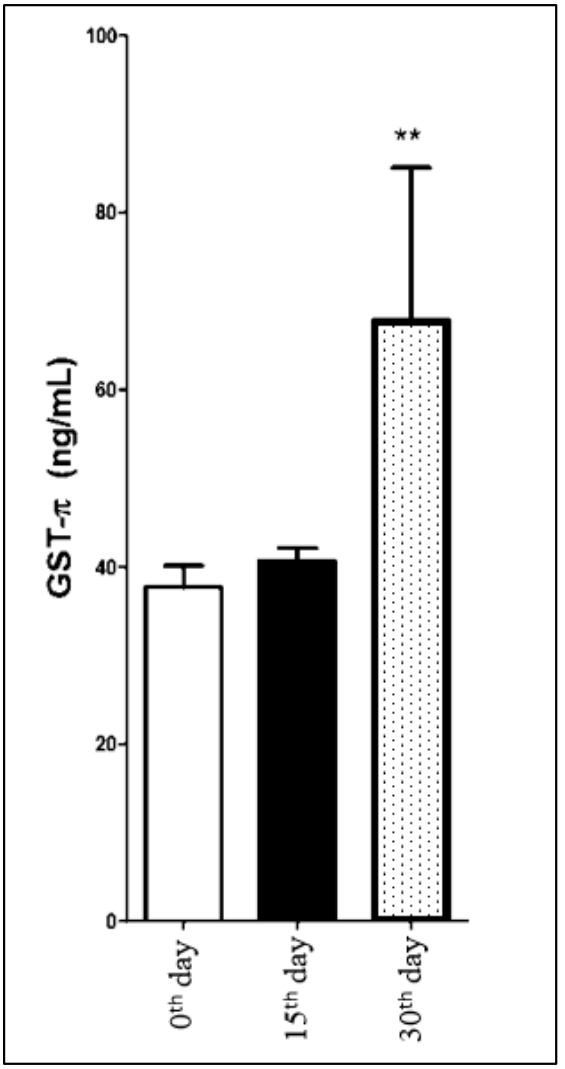

Figure 4. GST-π protein expression levels on 0th, 15th and 30th days (** P <0.01 versus 0th day)

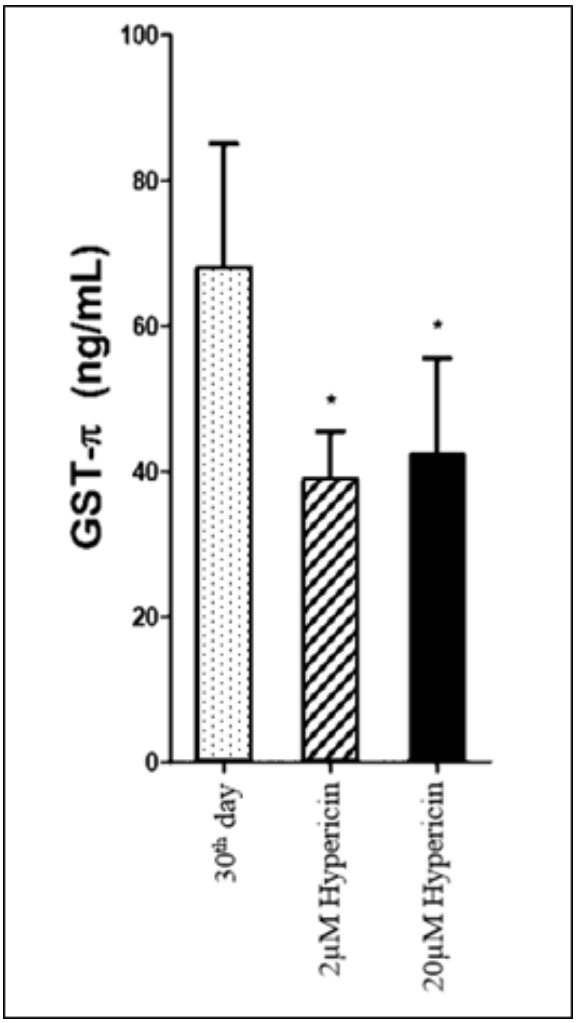

Figure 5. GST-π protein expression levels on 30th day after hypericin treatment (* P <0.05 versus 30th day)

4. Conclusion

Many tumor cells exhibit Epithelial-Mesenchymal Transition. Especially in these transitions, tumor cells in mesenchymal form show more resistant, invasive and poor prognosis than epithelial form. Epithelial cells of tumors acquire invasive and migratory properties, thus, this contributes to metastasis during carcinogenesis. Investigation of the EMT process and regulation may provide a better understanding of carcinogenesis. In this study, it was evaluated that GST levels in the mesenchymal form was higher than epithelial form which means the resistancy may be related to GSTs and inhibitor use could reverse the process to increase the effectiveness of treatment.

5. References

1. Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA: Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. The Journal of clinical investigation 2009, 119(6):1438-1449.
2. Boyer B, Valles AM, Edme N: Induction and regulation of epithelial-mesenchymal transitions. Biochemical Pharmacology 2000, 60(8):1091-1099.
3. Micalizzi D, Farabaugh S, Ford H: Epithelial-Mesenchymal Transition in Cancer: Parallels Between Normal Development and Tumor Progression. Journal of mammary gland biology and neoplasia 2010, 15:117-134.
4. Popov Z, Gil-Diez de Medina S, Lefrere-Belda MA, Hoznek A, Bastuji-Garin S, Abbou CC, et al.: Low E-cadherin expres-

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sion in bladder cancer at the transcriptional and protein level provides prognostic information. British journal of cancer 2000, 83(2):209-214.

5. Radisky DC: Epithelial-mesenchymal transition. Journal of cell science 2005, 118(Pt 19):4325-4326.
6. Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ, Yang J: Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. Cancer research 2011, 71(1):245-254.
7. Nieto MA: The snail superfamily of zinc-finger transcription factors. Nature reviews Molecular cell biology 2002, 3(3):155-166.
8. Zhao XJ, Li H, Chen H, Liu YX, Zhang LH, Liu SX, et al.: Expression of e-cadherin and beta-catenin in human esophageal squamous cell carcinoma: relationships with prognosis. World journal of gastroenterology 2003, 9(2):225-232.
9. Kang Y, Massague J: Epithelial-mesenchymal transitions: twist in development and metastasis. Cell 2004, 118(3):277-279.
10. Sayan AE: Tumour-promoting role of EMT-inducing transcription factor ZEB1 in mantle cell lymphoma. Cell death and differentiation 2014, 21(2):194-195.
11. Yao D, Dai C, Peng S: Mechanism of the mesenchymal-epithelial transition and its relationship with metastatic tumor formation. Molecular cancer research : MCR 2011, 9(12):1608-1620.
12. Armstrong RN: Structure, catalytic mechanism, and evolution of the glutathione transferases. Chemical research in toxicology 1997, 10(1):2-18.
13. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al.: The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008, 133(4):704-715.
14. Hayes JD, Flanagan JU, Jowsey IR: Glutathione transferases. Annual review of pharmacology and toxicology 2005, 45:51-88.
15. Laborde E: Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. Cell death and differentiation 2010, 17(9):1373-1380.
16. Aliya S, Reddanna P, Thyagaraju K: Does glutathione S-transferase Pi (GST-Pi) a marker protein for cancer? Molecular and cellular biochemistry 2003, 253(1-2):319-327.
17. Mannervik B, Board PG, Hayes JD, Listowsky I, Pearson WR: Nomenclature for mammalian soluble glutathione transferases. Methods in enzymology 2005, 401:1-8.
18. Noguti J, Barbisan LF, Cesar A, Dias Seabra C, Choueri RB, Ribeiro DA: Review: In vivo models for measuring placental glutatione-S-transferase (GST-P 7-7) levels: a suitable biomarker for understanding cancer pathogenesis. In vivo (Athens, Greece) 2012, 26(4):647-650.
19. Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, et al.: Regulation of JNK signaling by GSTp. The EMBO journal 1999, 18(5):1321-1334.
20. Lu WD, Atkins WM: A novel antioxidant role for liganding behavior of glutathione S-transferases: attenuation of the photodynamic effects of hypericin. Biochemistry 2004, 43(40):12761-12769.
21. Zhao L, Li C, Zhou B, Luo C, Wang Y, Che L, et al.: Crucial role of serum response factor in renal tubular epithelial cell epithelial-mesenchymal transition in hyperuricemic nephropathy. Aging 2019, 11.
22. Zhong X, Tang J, Li H, Shi X, Wu Y, Xia D, et al.: MiR-3175 promotes epithelial mesenchymal transition by targeting Smad7 in human conjunctiva and pterygium. FEBS letters 2019.
23. Chen T, You Y, Jiang H, Wang ZZ: Epithelial-mesenchymal transition (EMT): A biological process in the development, stem cell differentiation, and tumorigenesis. J Cell Physiol 2017, 232(12):3261-3272.
24. Tania M, Khan MA, Fu J: Epithelial to mesenchymal transition inducing transcription factors and metastatic cancer. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 2014, 35(8):7335-7342.
25. Giannoni E, Parri M, Chiarugi P: EMT and oxidative stress: a bidirectional interplay affecting tumor malignancy. Antioxidants & redox signaling 2012, 16(11):1248-1263.
26. Basu S, Chaudhary A, Chowdhury P, Karmakar D, Basu K, Karmakar D, et al.: Evaluating the role of hsa-miR-200c in reversing the epithelial to mesenchymal transition in prostate cancer. Gene 2019, 144264.
27. Turk S, Kulaksiz Erkmen G, Dalmizrak O, Ogus IH, Ozer N: Purification of Glutathione S-Transferase pi from Erythrocytes and Evaluation of the Inhibitory Effect of Hypericin. The protein journal 2015, 34(6):434-443.
28. Dabrowski MJ, Maeda D, Zebala J, Lu WD, Mahajan S, Kavanagh TJ, et al.: Glutathione S-transferase P1-1 expression modulates sensitivity of human kidney 293 cells to photodynamic therapy with hypericin. Archives of biochemistry and biophysics 2006, 449(1-2):94-103.
29. Dalmizrak O, Kulaksiz-Erkmen G, Ozer N: Evaluation of the in vitro inhibitory impact of hypericin on placental glutathione S-transferase pi. The protein journal 2012, 31(7):544-549.
30. Halder M, Chowdhury PK, Das R, Mukherjee P, Atkins WM, Petrich JW: Interaction of glutathione S-transferase with hypericin: A photophysical study. The journal of physical chemistry B 2005, 109(41):19484-19489.