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The Positive Correlation between The Expression of Endogenous Hydrogen Sulfide and Vascular Endothelial Growth Factor in Colorectal Cancer Tissues

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Keywords:

Hydrogen sulfide (H2S); Vascular endothelial growth factor (VEGF); Colorectal cancer (CRC); Correlation

Abbreviation:

H2S: Hydrogen Sulfide; CBS: Cystathionine-Beta-Synthase; CSE: Cystathionine-Gama-Lyase; 3MST: 3-Mercapto-pyruvate Sulfurtransferase; VEGF: Vascular Endothelial Growth Factor; CRC: Colorectal Cancer

1. Abstract

1.1. Objective

Hydrogen sulfide (H2S), a gas signal molecule, has been broadly studied in colorectal cancer (CRC), and is mainly regulated in vivo by three enzymes: CBS, CSE and MST. Tumor growth necessitates blood supply through angiogenesis, where the signaling cascade mediated by VEGF/VEGFR2 modulate the proliferation, migration and survival of vascular endothelial cells and vascular permeability. Animal studies demonstrate that H2S can up-regulate the expression of VEGF in tumor growth. Thus, we aim to investigate the correlation of the factors in human CRC tissues.

1.2. Methods

Briefly, 90 cases of CRC and paired adjacent non-tumor tissues were prepared and embedded accordingly for IHC staining. Quantitative evaluation of IHC staining for CBS, CSE and VEGF between the paired two groups was designed and completed by two professional and experienced pathologists. The data were described as mean +/- standard deviation. Paired t-test was adopted to compare the means between the matched two groups, and linear correlation using Pearson coefficient to investigate the correlation between H2S and VEGF. Significance was set to 0.05.

1.3. Results

The epitomes of CBS, CSE and VEGF IHC positive stainings were illustrated. Quantitative analyses showed that IHC staining scores of CBS, CSE and VEGF in CRC were significantly higher than that in paired adjacent non-tumor tissues (8.911 +/- 2.573 vs. 6.267 +/- 2.207, $p<0.05$; 9.167 +/- 2.029 vs. 6.311 +/- 1.882, $p<0.05$; and 6.81 +/- 3.69 vs. 6.00 +/- 3.23, $p<0.05$, respectively). The correlations between CBS and VEGF/CSE and VEGF IHC scores in 90 CRC cases were calculated to be $r= 0.399$ with $P<0.05$ and $r=0.451$ with $P< 0.05$, respectively.

1.4. Conclusion and Discussion

The CBS, CSE and VEGF in CRC tissues were elevated, and the linear correlations between CBS/CSE and VEGF were established. Yet, a cause and effect relationship between CBS/CSE and VEGF still needs advanced studies to provide conclusive evidences. Based

on the current data of this study, H2S might play an important role in CRC growth and migration.

2. Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies, causing heavy health burden around the world [1, 2]. Although the advances in treatment have triggered survival improvement in the early stage of CRC, the prognosis of the advanced stage is still poor, thus, posing a challenge to investigate the potential factors that affect the progression of the tumor. Hydrogen sulfide (H2S) has a similar effect to Nitric oxide (NO) and Carbon monoxide (CO), and is involved in the pathophysiological processes of various diseases and considered as the third class of gas signaling molecules in human body [3]. There are three well-studied enzymes: cystathionine-gama-lyase (CSE), cystathionine-beta-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3MST), activizing the production of H2S in different tissues in vivo [4]. In brain tissues CBS mainly generates H2S, in peripheral tissues CSE catalyzes the production of H2S, and both enzymes exist in the brain and peripheral tissues. In addition, the third enzyme 3MST leading to biosynthesis of H2S also play a role in brain tissues [5]. H2S, a lipophilic compound, is feasible of penetrating cell membranes and entering various cell types easily, and plays a pivotal role in various physiological and pathological functions [6, 7]. Studies have presented that the levels of CBS, CSE or 3MST enzymes and the productions of H2S are higher in various tumors tissues of colon, breast, ovarian and prostate as compared to adjacent non-tumor tissues [8, 9]. VEGF, a strong angiogenesis factor, binds specifically to VEGF receptor 1 and receptor 2 on vessel endothelium, promoting endothelium regeneration and tumor angiogenesis, vascular permeability and remodeling [9]. It has been documented that VEGF is highly expressed in CRC tissues and have become a treatment target in cancer [10]. Given the H2S can functionally act on blood vessel, we hypothesize that the production of H2S may play a role in CRC angiogenesis via a mechanism connected with the expression of VEGF.

3. Materials and Methods

3.1. Sample Preparation for IHC and Research Subjects

90 clinical cases of CRC surgical samples were prepared for IHC. All 90 subjects were from the sample bank of our hospital with signed consent forms for future clinical research. Briefly, two groups of CRC and adjacent non-tumor tissues were fixed by formaldehyde, separately, and then embedded in paraffin. A microtome was used to section the tissue, which would then be dried onto microscope slides and stored at room temperature. Once antigen retrieval and permeabilization were done, goat serum was used to block non-specific binding. The antibodies of CBS, CSE and VEGF, purchased from Themo Fisher Scientific, were incubated with the sections to bind the proteins of interest according to the manufacturer's instruction. At last, the DAB detection system was applied to dye the binding components in brown color. Counterstaining of nucleus was in blue.

3.2. Quantitative Assessment of IHC Positive Staining

The proteins of interest were evaluated quantitatively in IHC staining. The quantitative method was designed by two professional pathologists in the field of CRC. Specifically, various colors were assigned different scores based on the pathologists' experiences: 0 - no indicating color; 1 - light yellow; 2 - yellow; 3 - yellowish-brown or brown. The two pathologists assessed the section slides separately to avoid errors. If the discrepancy of assessed scores were more than 2 points, a third pathologist would be consulted to assess the sample again.

3.3. Statistics

SPSS 26.0 was applied to perform and draw all the analyses and scatter plots. The sample sizes were 90 cases, sufficiently large to follow normal distribution according to the central limit theorem. Thus, numerical data was described as mean +/- standard deviation. Paired-t test was adopted to compare the means of IHC scores between CRC and its paired adjacent non-tumor tissues. Linear correlation analysis, choosing Pearson coefficient, was used to investigate the correlation between CBS/CSE and VEGF. And the bar graphs with mean and SD were drawn by using Graphpad Prism 10. The statistical power was set to 0.05.

4. Results

4.1. The Epitome of CBS, CSE, and VEGF IHC Stainings and the Characteristics of the 90 Cases

The IHC stainings of CBS, CSE, and VEGF in CRC and paired adjacent tissues of typical case were illustrated (Figure 1). The yellow or yellow-brown color on CRC chip indicated positive staining of CBS, CSE and VEGF, while on paired adjacent non-tumor tissues the color was dyed mainly in blue. The discrepancy in yellow or yellow-brown indicated that the expression of the three factors were higher in the CRC tissues as compared with the paired adjacent non-tumor tissues. And the characteristics of the 90 CRC clinical cases were summarized (Table 1).

Table 1: Characteristics of the 90 CRC cases.

Clinical and Pathological information	Number
Age	
<60	46
≥60	44
Sex	
Male	56
Female	34
TNM	
I	7
II	40
III	40
IV	3
Pathological Grades	
G1	8
G2	53
G3	26
G4	3
Total	90

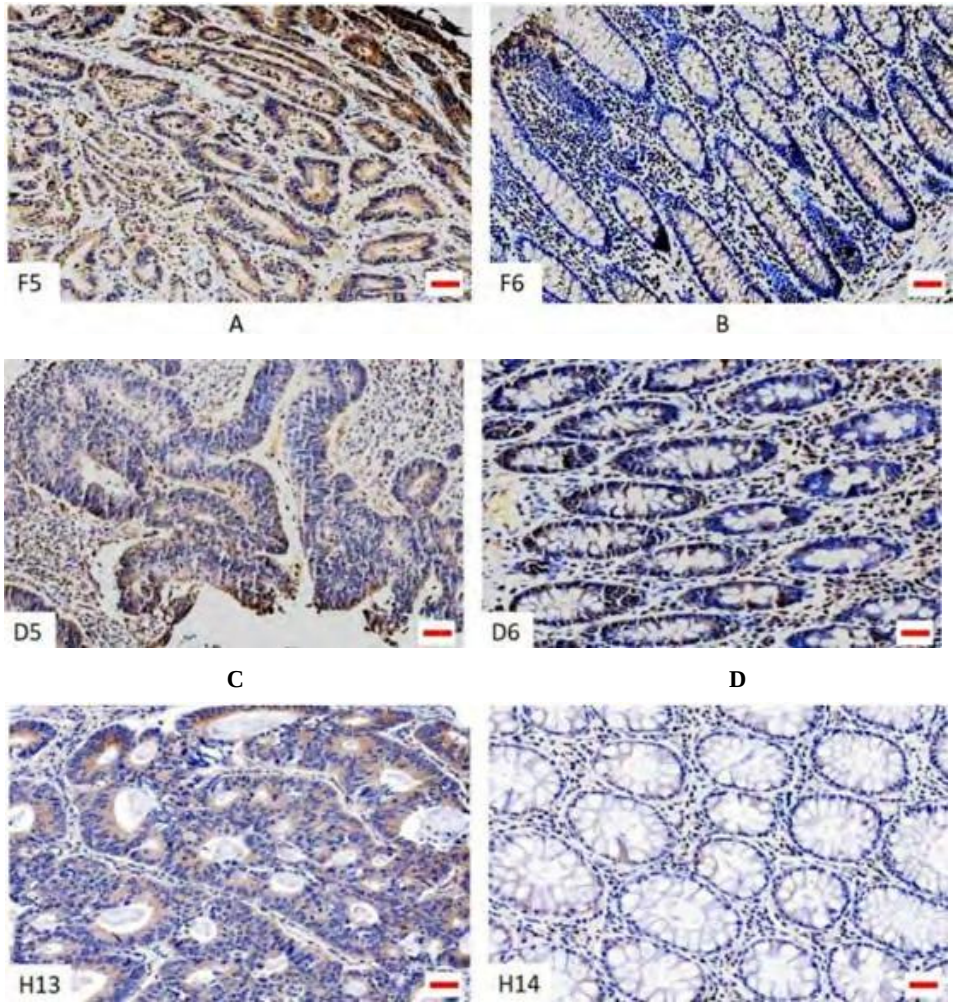

Figure 1: Illustration of the epitome of CBS, CSE and VEGF positive IHC staining (x 400 times). The paired F5 and F6 on the left bottom of slides A and B came from the same case assigned as F (supplement data), representing CBS staining in CRC and paired adjacent non-tumor tissues, respectively; the paired D5 and D6 from the case D, demonstrating CSE staining of the two groups, orderly, and paired H13 and H14 from the case H, showing VEGF staining from the two groups, sequentially. A, C, E of CRC tissues illustrated yellowish or brown color of positive dyeing as compared with B, D, F of adjacent non-tumor tissues with mainly blue counterstains.

4.2. Quantitative Analyses of CBS, CSE and VEGF IHC Positive Staining Scores in CRC Tissue and Adjacent non-Tumor Tissue

The quantitative results showed that the mean +/- standard deviation of CBS, CSE and VEGF positive staining scores in CRC compared with adjacent non-tumor tissues were 8.911 +/- 2.573 vs. 6.267 +/- 2.207, 9.167 +/- 2.029 vs. 6.311 +/- 1.882, and 6.81 +/- 3.69 vs. 6.00 +/- 3.23, respectively. The paired t-test adopted indicated that the scores of CBS, CSE and VEGF in CRC were significantly higher than in adjacent non-tumor tissues (all $P<0.05$) (Figure 2).

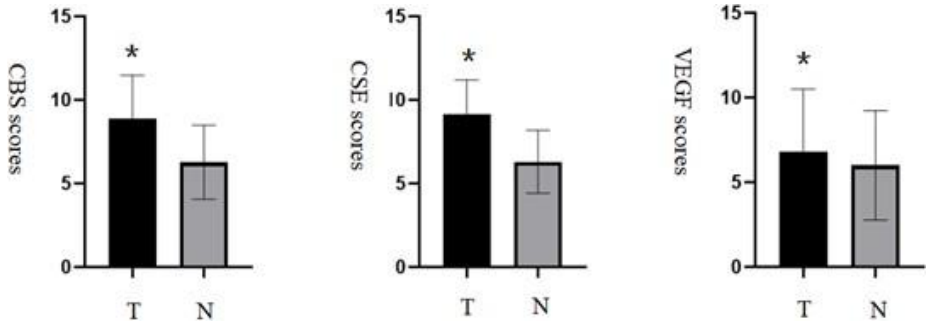

Figure 2: Bar graphs of CBS, CSE, and VEGF scores between *T CRC* and *N adjacent non-tumor tissues* (mean +/- SD). The bar graphs demonstrated *T CRC* scores of CBS, CSE and VEGF were significantly higher than *N adjacent non-tumor tissues* scores marked out by ' * ' (8.911 +/- 2.573 vs. 6.267 +/- 2.207, $p<0.05$; 9.167 +/- 2.029 vs. 6.311 +/- 1.882, $p<0.05$; 6.81 +/- 3.69 vs. 6.00 +/- 3.23, $p<0.05$, respectively.).

4.3. Identical IHC Positive Stainings of CBS to VEGF, and CSE to VEGF

Consistent IHC stainings of CBS with VEGF, and CSE with VEGF in typical cases were exemplified, sequentially (Figure 3). While the adjacent non-tumor slides showed negative or less stainings of CBS, VEGF was also weakly dyed in the adjacent non-tumor tissues of the same case. By contrast, the case of CRC with elevated CBS staining also demonstrated a strong VEGF expression. And the same pattern applied to CSE and VEGF in both tissues. This results indicated that the CBS and CSE elevation were conforming with VEGF high expression in CRC.

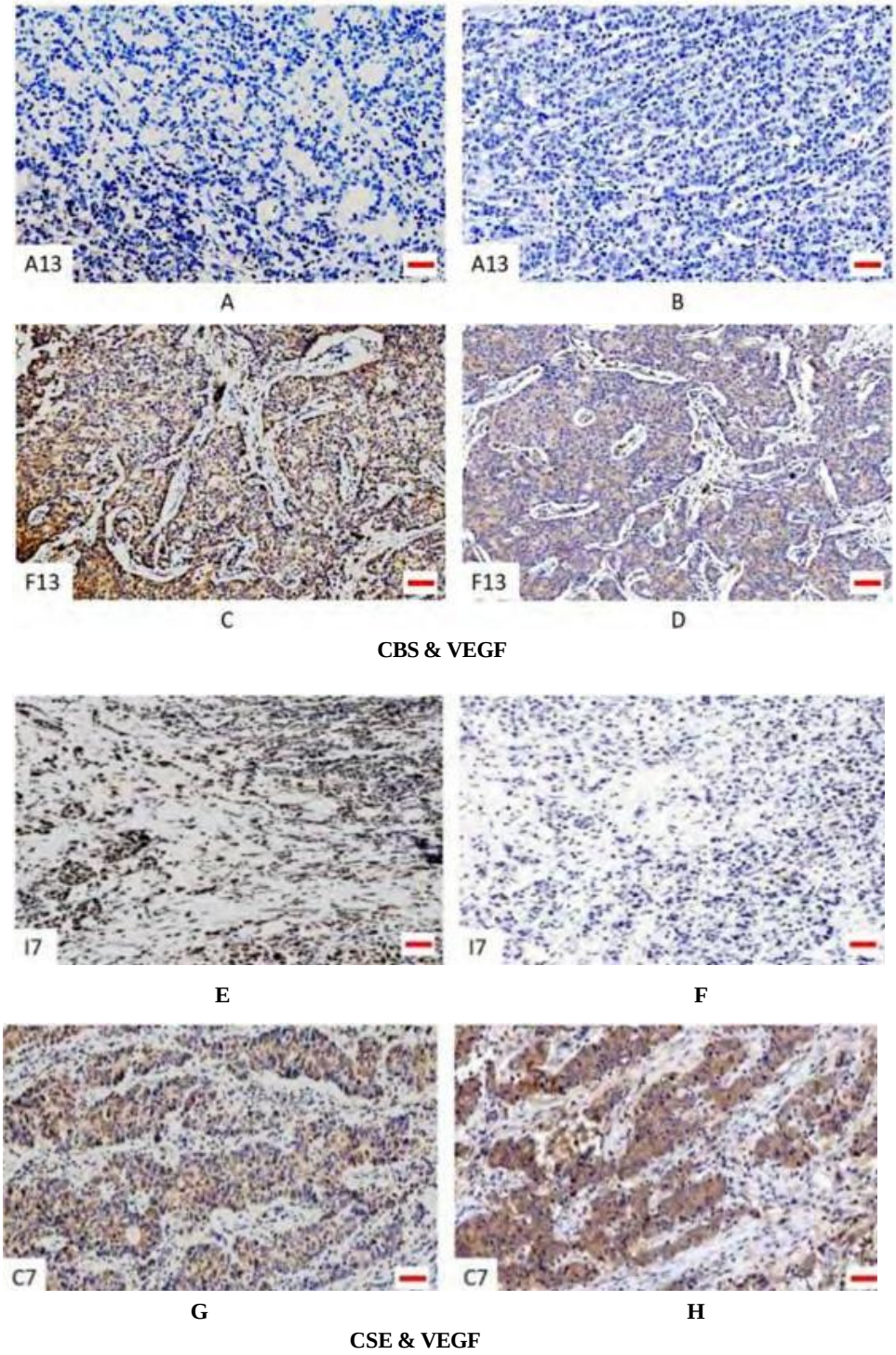

Figure 3: Similar stainings between CBS and VEGF/CSE and VEGF. *A and B* were the adjacent non-tumor tissues from the case 13, and *C and D* of the case were CRC tissues. *A CBS and B VEGF* showed accordingly blue color, while *C CBS and D VEGF demonstrated* positive brown stainings in the same pattern. The *E, F, C, and D* were from another case 7 with *E and F* being the adjacent non-tumor tissues and *G and H* CRC tissues. While E CSE and F VEGF were stained in less brown, G CSE and H VEGF revealed strong brown color

4.4. Correlation Between CBS and VEGF/CSE and VEGF in 90 CRC Cases

The scatter plots were graphed, displaying linear trends between CBS and VEGF/CSE and VEGF. (Fig.4). Pearson product-moment correlation was adopted. The correlation between CBS and VEGF IHC scores in 90 CRC cases was calculated to be r= 0.399, P<0.05. And the correlation between CSE and VEGF was r=0.451, P< 0.05. These results indicated the correlation was positive between CBS and VEGF, and so was between CSE and VEGF.

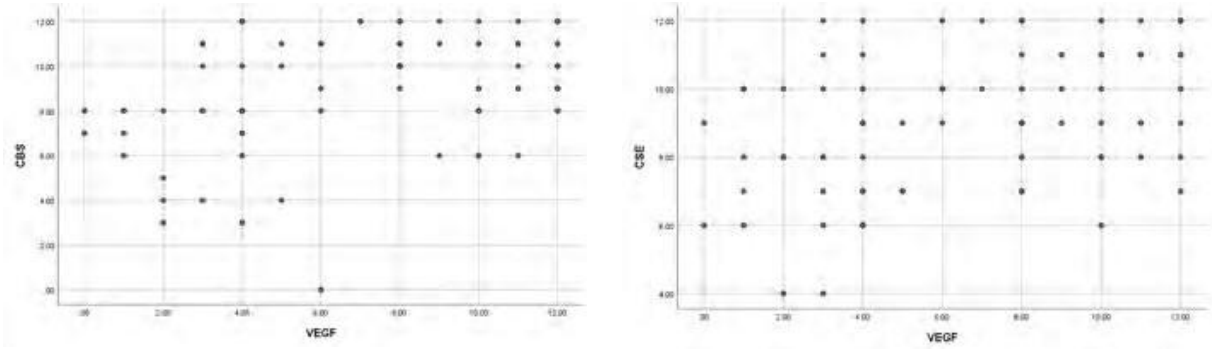

Figure 4: Scatter plots. The linear trends were displayed between CBS and VEGF in *A,* and between CSE and VEGF in *B.*

5. Discussion

H2S is a lipid affinity molecule that can penetrate cell membrane easily without a transporter, and endogenously plays various important physiological functions in human body [11]. Pathogenesis of cancer can attribute to potential decreasing of H2S [12]. Its synthesis in humans mainly comes from two sources: enzymatic generation and metabolic production through bowel bacteria [13, 14]. The enzymatic pathway of H2S production involves L-cysteine, an amino acid synthesized inside human body, and three enzymes: CBS, CSE, and 3MST. In neuro system, CBS acts as a predominant enzyme in producing H2S, and is also expressed in other organs of ileum, liver, kidney, pancreas, uterus, placenta, and vascular system [15, 16]. Human CBS, a homopolymer, generates H2S in three distinct pathways: hydrolyzing cysteine to serine and H2S, condensing cysteine and homocysteine to cystathionine and H2S, and forming H2S and lanthionine through two cysteine molecules reaction [16, 17]. CSE, capable of producing H2S through cysteine and homocysteine, is also expressed in ileum, liver, pancreas, kidney, uterus, placenta, and vascular system [18]. It catalyzes molecules in a similar mechanism to CBS. 3MST, catalyzing sulfur transfer from sulfur donor of 3-mercaptopyruvate to form cysteine persulfur intermediate, belongs to the sulfurtransferase family [19]. The persulfide intermediate reacts with other molecules and eventually produces H2S [20]. In addition, transsulfuration of L-methionine can also produce H2S, acting as a protector factor in cardiovascular and neurocognitive diseases [21]. In this study of CRC, we mainly detected CBS and CSE expressions via IHC staining technique, and found that the levels of CBS and CSE were much higher in CRC tissues than that in adjacent non-tumor tissues. This results indicated that H2S might be produced in a large amount corresponding to tumor growth. Tumor Angiogenesis plays a pivotal role in malignancy progression [22]. To suppress tumor vessel growth can lead to tumor cell dormant and control the progression of tumor [23]. In CRC, targeting vessel growth related factors can prolong the survival of patients [10]. Consequently, anti-angiogenic therapy has been established in this lethal tumor. Although antibodies and tyrosine kinase inhibitors have been designed and approved in clinical practice by FDA, the anti-cancer effects have been observed to be compromised, providing only short-term of relief from tumor growth in monotherapy. Disappointedly, tumor resistance can be developed after the initialization of the monotherapy [24]. The compromised efficacy has so far been thought to be related to the molecular and functional heterogeneities of tumor vessels contributing to alternative vascularization mode [25]. VEGF has long been studied to be noted to play a significant role in tumor angiogenesis. Tumor cells can secrete VEGF acting on endothelial cells, promoting vessel formation, increasing vascular permeability and resulting tumor cell progression via specific mechanisms [26]. We explored the promotion of VEGF in human CRC tissues through in-situ IHC staining, which showed that the levels of VEGF in CRC tissues were much higher than in adjacent non-tumor tissues by quantitative scoring approaches. Consistent with established ideas of VEGF in CRC, the results indicated that the expression of VEGF had been largely elevated in the CRC samples of our study.

H2S is noted as the third gaseous signaling, apart from NO and CO, being cable of affecting the physiological functions of vascular in human body [3]. In consideration of its role in cancer and CRC in particular, we progressively formed the idea that it would be of interest in exploring the correlation between H2S and VEGF in the setting of CRC, since these two factors can both affect the physiological and pathological functions of vascular in both healthy and diseased human body. Using the quantitative data of IHC scoring evaluated by two professional pathologists, our study demonstrated a positive correlation between H2S and VEGF. Notably, this linear trend in correlation analysis doesn't imply causation. Whether the changes in H2S in the setting of CRC cause the changes of VEGF still needs further studies to rule out all potential factors. In sum, the positive

correlation provides a clue for advanced studies to explore the causality of those two factors.

The limitations of our study include that the causality of H2S and VEGF elevation in the setting of CRC needs more data to rule out all other potential factors before reaching a strong conclusion. Additionally, in this study, the evaluation of H2S was based on indirect detection methods, assessing the two enzymes required in the production of the gaseous factor. Developing direct detection of H2S might be more accurate in advanced studies, leading to potential and significant findings.

As mentioned above, anti-angiogenic therapies have been proved effective in treating CRC. VEGF targeting antibody has been approved in the treating, yet the resistance can be soon developed to compromise the effect of the monotherapy. Heterogeneous vessel phenotype might be attributed to the failing. In this respect and with the above findings, H2S, as a vessel functioning factor, might help explain the non responding and/or resistance mechanism developed in the anti-angiogenic therapy for CRC, thus, developing a novel therapeutic targeting point and optimize the anti-tumor combination therapy.

6. Funding

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7. Ethical Approval

Ethical approval and participant signed consent form have been received for this study. The study has followed the guidelines of the Helsinki Declaration.

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