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大腸早期がんの診断とESD/EMRの選択、特別講演、口演	斎藤豊	山口グランドホテル２Ｆ「鳳凰の間」、山口市	2014/11/19	国内
大腸腫瘍の内視鏡診断とEMR/ESD-NBI拡大分類も含めて-、特別講演、口演	斎藤豊	第3回東海大腸腫瘍セミナー、キャスルプラザホテル3Ｆ［孔雀の間」、名古屋市	2014/11/27	国内
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（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。
（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

消化管がんに対する特異的蛍光内視鏡の開発とその臨床応用に向けた研究

平成２６年度

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A Pilot Study of Fluorescent Imaging of Colorectal Tumors Using a γ-Glutamyl-Transpeptidase-Activatable Fluorescent Probe

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Key Words
Activatable fluorescent probe · Colorectal tumor · Fluorescent imaging · γ-Glutamyl hydroxymethyl rhodamine green · γ-Glutamyl-transpeptidase

Abstract
Backgrounds/Aim: Colorectal laterally spreading tumors (LSTs) are sometimes difficult to visualize even with image-enhanced endoscopy. γ-Glutamyl-transpeptidase (GGT) is a cell surface-associated enzyme that is overexpressed in various types of human cancers. Furthermore, GGT expression is higher in colorectal cancer cells than in normal colorectal mucosa. γ-Glutamyl hydroxymethyl rhodamine green (gGlu-HMRG), an activatable fluorescent probe, is nonfluorescent under a neutral pH and normal cellular environment; however, it turns highly fluorescent upon reaction with GGT. We evaluated ex vivo fluorescent imaging of colorectal LSTs using this GGT-activatable fluorescent probe. ***Methods:*** Between March 2013 and March 2014, 30 endoscopically resected colorectal LSTs were prospectively included in this study. Each was analyzed by first taking a baseline image before spraying, then spraying with gGlu-HMRG onto the freshly resected specimen, and finally taking fluorescent images 15 min after spraying with a dedicated imaging machine. ***Results:*** Of the LSTs, 67% rapidly showed positive fluorescent activity. These activities were shown in adenoma (54%) and carcinoma in adenoma (76%), and in LST-granular type (80%) and LST-nongranular type (40%). ***Conclusion:*** Topically spraying gGlu-HMRG enabled rapid and selective fluorescent imaging of colorectal tumors owing to the upregulated GGT activity in cancer cells.

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Introduction

Currently, screening colonoscopy is widely accepted as the gold standard for colorectal cancer detection [1–3]. Indeed, early detection of polyps and their subsequent endoscopic removal are the best ways to reduce colorectal cancer mortality [1–3]. Various image-enhanced endoscopy techniques, such as chromoendoscopy [4, 5], narrow-band imaging [6, 7], autofluorescence imaging [8] and confocal laser endomicroscopy [9], have improved the detection and characterization of colorectal neoplasms. However, laterally spreading tumors (LSTs) are still difficult to detect with such modalities [10–14]. LST-nongranular type (LST-NG) is particularly difficult to visualize even with a large tumor size, which has a higher potential risk of lymph node metastasis and should be treated by endoscopic submucosal dissection (ESD) [14–16].

Optical fluorescence molecular imaging has been investigated for optically guided surgery and endoscopy [17]. γ-Glutamyl-transpeptidase (GGT) is a cell surface-associated (or -bound) enzyme involved in cellular glutathione homeostasis, and is considered to play a role in tumor progression, invasion and drug resistance [18]. GGT is poorly expressed in normal tissue, but overexpressed in vivo on the cell surface membrane of various cancer cells, such as cervical and ovarian cancers [19–21].

Urano et al. [22] developed an enzymatically activatable fluorescent probe, γ-glutamyl hydroxymethyl rhodamine green (gGlu-HMRG) in vivo, which originated from the fluorophore rhodamine green that became fluorescent after cleavage of a GGT-specific sequence. gGlu-HMRG is nonfluorescent under neutral pH and a normal cellular environment with low GGT activity. When gGlu-HMRG reacts with GGT on the surface of a cancer cell, gGlu-HMRG is promptly hydrolyzed and transformed into HMRG, showing strong fluorescence, and permeates the plasma membrane and accumulates in the lysosomes of cancer cells. Furthermore, topically spraying gGlu-HMRG could provide immediate and specific enhancement of the cells overexpressing GGT [22, 23]. Therefore, this activatable fluorescent probe may be a new modality for cancer-selective fluorescent imaging. However, only limited data from mice models have been reported [22–24]. We assumed that gGlu-HMRG might be applied to the visualization of a superficial colon neoplasm with high GGT activity, leading us to conduct this pilot study [25]. The study aimed to evaluate ex vivo fluorescent imaging of colorectal tumors using the GGT-activatable fluorescent probe.

Materials and Methods

Lesion Inclusion Criteria

Between March 2013 and March 2014, a total of 30 endoscopically resected LSTs at the National Cancer Center Hospital in Tokyo, Japan, were prospectively included in this study. Clinical macroscopic type was divided into LST-granular type (LST-G) and LST-NG. LST-G is defined by the presence of aggregates of even or uneven nodules on the surface, and LST-NG has a smooth surface lacking granular formations [26, 27]. The right-side colon was defined as including the lesions located in the cecum, ascending colon and transverse colon. The left-side colon was defined as a descending colon and sigmoid colon.

Imaging Method of Fluorescent Imaging

We used a handheld fluorescent imaging machine (Discovery; INDEC Inc., Santa Clara, Calif., USA) which could provide the still images under white light and 450–490 nm of blue excitation light (fig. 1a). The freshly resected specimens were quickly fixed on a black board and baseline images were obtained before spraying gGlu-HMRG by blue light and white light with the Discovery machine. Then, 1,000 μl of gGlu-HMRG in a concentration of either 50 or 500 μM were topically sprayed onto the resected specimen. We first used gGlu-HMRG in a concentration of 500 μM based on previous reports, and then modified this to 50 μM [22–24]. After spraying, fluorescent images were subsequently taken every 30 s for 15 min.

Assessment of Fluorescent Image with gGlu-HMRG

The fluorescent images were evaluated by three endoscopists. The fluorescent activity was determined to be positive when the lesion was illuminated after spraying gGlu-HMRG compared with the baseline image, even if the illumination was partial or heterogeneous. The activity was determined to be negative if the illumination showed no difference from that of the baseline image.

Histopathological Assessment

After fluorescent imaging all resected specimens were fixed in 10% buffered formalin and cut into 2-mm slices. These specimens were embedded in paraffin, cut into 3-μm sections, stained with hematoxylin and eosin, and microscopically examined for histopathological diagnosis. Experienced gastrointestinal pathologists assessed the macroscopic and histological type, tumor size, depth of invasion, lymphovascular invasion and resected margin according to the Japanese Classification of Colorectal Carcinoma [28]. The pathological findings were evaluated with the nonneoplastic, adenomatous or cancerous area, and the distribution of the adenoma and/or cancer was shown on the mapping. We evaluated the consistency between the illuminated area in the fluorescent image and the tumor extension in the pathological mapping.

Results

Clinicopathological Features

The mean age was 68 ± 7 years and the study included 15 male and 15 female patients. The mean tumor size was 39 ± 13 mm, 20 were LST-G and 10 were LST-NG macroscopic type. Thirteen lesions were diagnosed as adenoma and 17 were diagnosed as carcinoma in adenoma (table 1).

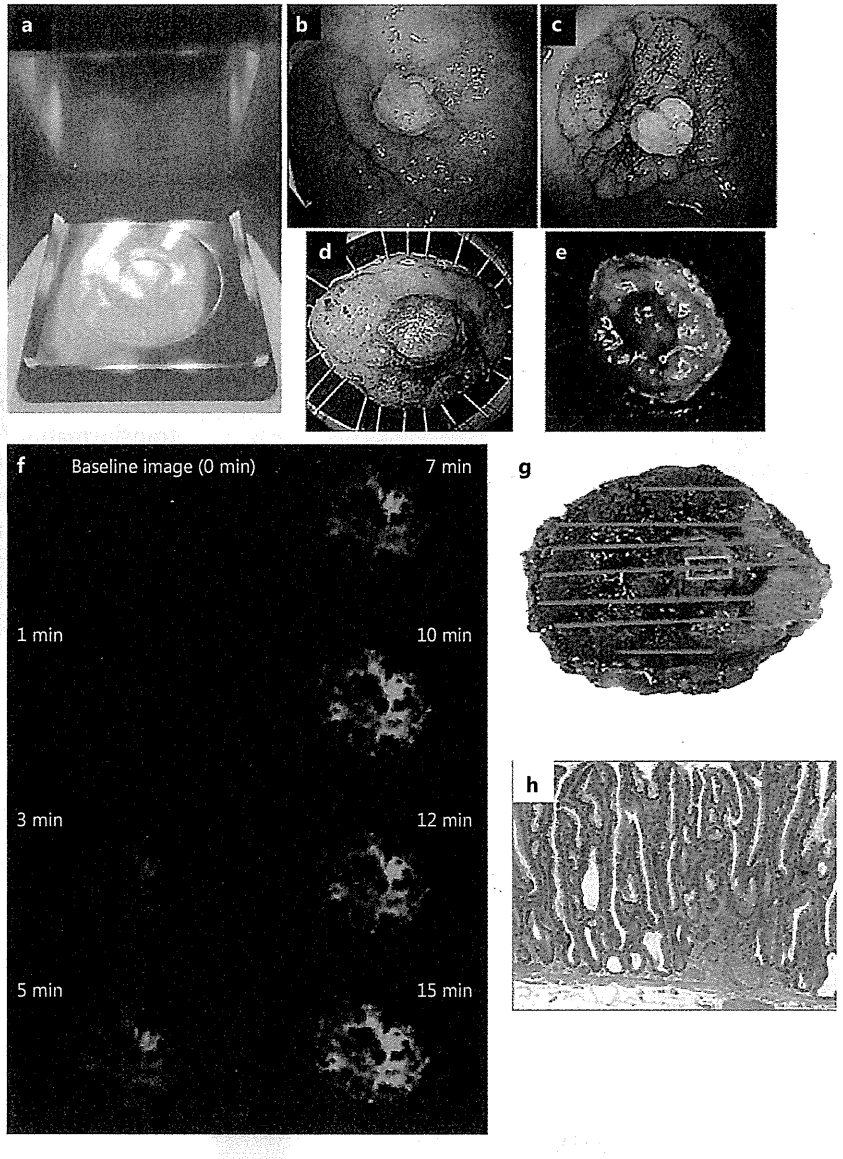

Fig. 1. a The Discovery machine (INDEC Inc.), which provides fluorescent images (emission wavelength >520 nm by long-pass filter) by blue-light (450–490 nm) excitation, with which the baseline and fluorescent images were taken. **b**, **c** White-light image and chromoendoscopic image: the endoscopic diagnosis was Ra, 0-Is+IIa (LST-G), 40 mm in size, cTis. **d** Endoscopic white-light image of a resected specimen. **e** White-light image of a resected specimen taken by Discovery. **f** Fluorescent images after spraying gGlu-HMRG with blue light; rapid fluorescent increase was observed in the tumor regions. **g** Purple lines on histological mapping showed tubular adenocarcinoma. **h** Histological finding of the blue box on the histological mapping indicated colonic well-differentiated tubular adenocarcinoma. HE stain. ×40.

Fluorescent Activity

Twenty (67%) of the images were fluorescently positive for the tumor region after 15 min, and 10 (33%) were negative. The mean tumor sizes in the positive and negative groups were 42 and 32 mm, respectively. Of the 13 adenomas, 7 (54%) lesions were positive and 6 (46%) were negative. Of the 17 carcinomas in adenomas, 13 (76%) lesions showed positive fluorescence and 4 (24%) were negative. According to the macroscopic type, 16 LST-G lesions (80%) and 4 LST-NG lesions (40%) were positive (table 2). Eighteen (75%) of 24 lesions were positive in 50 μM gGlu-HMRG, and 2 (33%) of 6 lesions were positive in 500 μM. Among the 17 carcinomas in adenoma, 12 (80%) of 15 lesions were positive in 50 μM and 1 (50%) of 2 lesions was positive in 500 μM. No histological tissue damage by gGlu-HMRG was seen in any of the resected specimens. Of the 20 positive lesions, some showed a heterogeneous fluorescent increase in the tumor region. An example case was an LST-G lesion 40 mm in size located in the upper rectum. This lesion was removed by ESD. The resected specimen was fixed on a black board and baseline images were obtained. A rapid and heterogeneous fluorescent increase was

Table 1. Clinicopathological characteristics of colorectal tumors (n = 30)

Age, years	68 ± 7
Sex	
Male	15
Female	15
Location	
Right colon	15
Left colon	9
Rectum	6
Endoscopic treatment	
ESD	27
Endoscopic mucosal resection	3
Size, mm	39±13
Macroscopic type	
LST-G	20
LST-NG	10
Histology	
Adenoma	13
Carcinoma in adenoma	17
GGT fluorescent activity	
Positive	20 (67)
Negative	10 (33)

Values are mean ± SD or number with percentage in parentheses.

Table 2. Fluorescent imaging of colorectal lesions with gGlu-HMRG

	Positive (n = 20)	Negative (n = 10)
Size, mm	42±11	32±1
Macroscopic type		
LST-G (n = 20)	16 (80)	4 (20)
LST-NG (n = 10)	4 (40)	6 (60)
Histology		
Adenoma (n = 13)	7 (54)	6 (46)
Carcinoma in adenoma (n = 17)	13 (76)	4 (24)

Values are mean ± SD or number with percentage in parentheses.

observed in the tumor regions after spraying gGlu-HMRG. The histological diagnosis was of a well-differentiated tubular adenocarcinoma, pTis, ly0, v0, VM0, HM0 (fig. 1b–h). One 40-mm LST-G lesion located in the cecum was also removed by ESD. This lesion had a positive fluorescent activity in a tiny area after spraying gGlu-HMRG. The histological diagnosis was of a well-differentiated tubular adenocarcinoma in adenoma, pTis, ly0, v0, VM0, HM0, and the distribution of the adenocarcinoma component on histological mapping was approximately consistent with the tiny area of positive fluorescent activity (fig. 2).

Discussion

GGT expression is considered to be associated with a 'resistance phenotype' exhibited by preneoplastic transformed cells [18]. GGT is overexpressed in various human cancer types in vivo [19–21] and is therefore considered to be a potential biomarker for early cancer detection. This association between GGT and neoplastic transformation was highlighted in several experimental models of chemical carcinogenesis in laboratory animals [18]. The previous literature showed that GGT activity was organ dependent, and the activity was high in several cancer cells, such as the lung, ovary, liver and bile duct [19–21]. Urano [29] reported that GGT fluorescent activity was significantly enhanced to about 60% of the cultured cancer cells. Mitsunaga et al. [23] also reported that gGlu-HMRG could improve endoscopic detection of colitis-associated colon cancer in a mouse model of ulcerative colitis.

In this study, we successfully observed tumor regions as being fluorescently positive by topically spraying of gGlu-HMRG. This method showed a rapid and specific fluorescent increase upon reaction with GGT without any histological tissue damage in an ex vivo model. This is a valuable finding relating to the ex vivo fluorescent imaging of human colorectal LSTs.

Generally, two major categories of fluorescent probes have been used in molecular imaging: 'always-on' and 'activatable'. Always-on probes, such as positron emission tomography, have the disadvantage of high background signal, which requires considerable time to clear to achieve adequate target-to-background ratios. On the other hand, several activatable probes, such as cathepsins, have lower background signal, but the activation process often requires hours to days, impeding the practicality for real-time clinical use in endoscopy [30].

In the present study, overall GGT fluorescent activity was positive in 67% of the specimens. Lesions with a carcinoma component were characterized by higher fluorescence than those with an adenoma component alone, which might imply that there was a relationship between the malignant potential of the cells and GGT fluorescent activity. In addition, some lesions with positive GGT activity revealed heterogeneous fluorescent activity (fig. 1b–h). This might be associated with a histological heteroge-

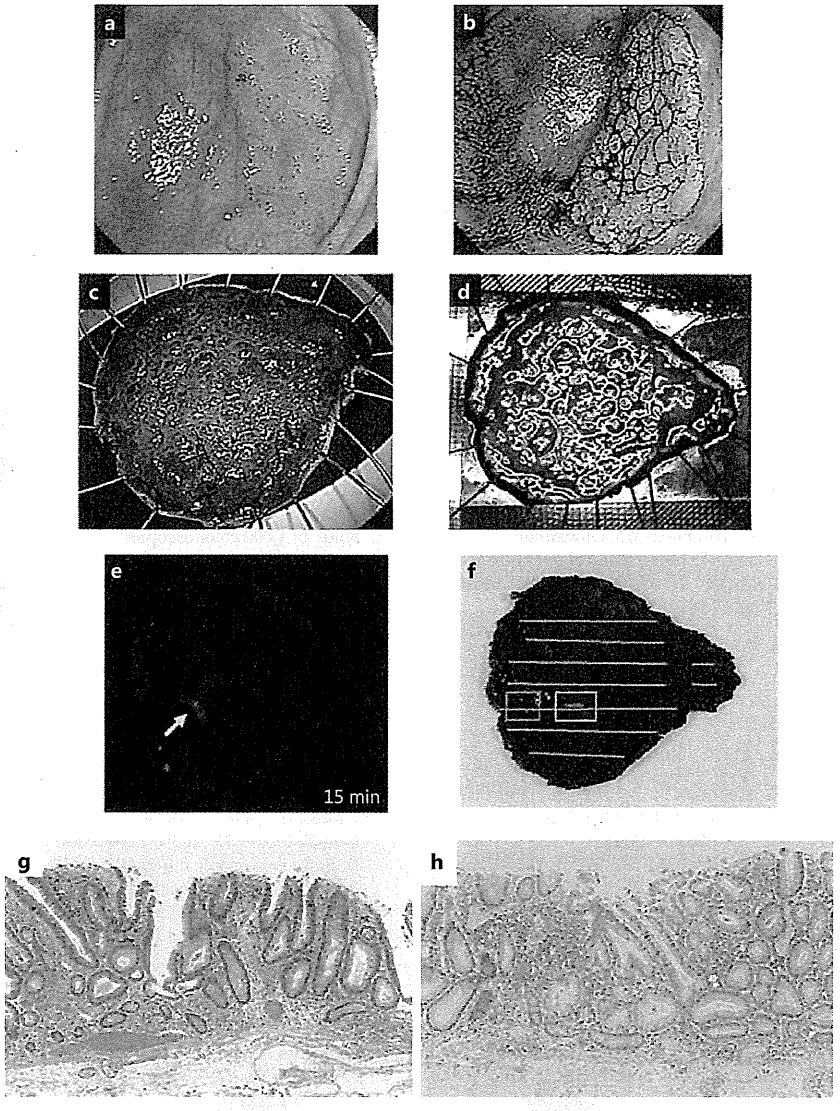

Fig. 2. a, b White-light image and chromoendoscopic image: the endoscopic diagnosis was cecum, 0-IIa (LST-G), 40 mm in size, cTis. **c** Endoscopic white-light image of a resected specimen. **d** White-light image of a resected specimen taken by Discovery. **e** Fluorescent images after spraying gGlu-HMRG with blue light; positive fluorescent activity was seen in a tiny area, as shown by the yellow arrow. **f** Histological mapping: the green lines show tubular adenoma and the narrow range of purple lines show tubular adenocarcinoma. **g** Histological finding of the blue box on the histological mapping indicated colonic adenoma. HE stain. ×40. **h** Histological finding of the yellow box on the histological mapping indicated colonic well-differentiated tubular adenocarcinoma. The distribution of the adenocarcinoma component on histological mapping was approximately consistent with a partially positive GGT fluorescent activity. HE stain. ×40.

neity between adenoma and adenocarcinoma due to the well-known adenoma-carcinoma sequence. Some lesions showed partial GGT fluorescent activity and we experienced an interesting case of intramucosal adenocarcinoma in adenoma (fig. 2). This case showed that the partially positive fluorescent activity and positive area was approximately consistent with the adenocarcinoma component.

This was a pilot study using ex vivo specimens and did not show significant clinical features associated with positive fluorescent imaging by gGlu-HMRG spraying. In addition, the study used two gGlu-HMRG concentrations of 50 and 500 μM. Of 17 carcinomas in adenomas, the proportion of positive fluorescent activity in 50 and 500 μM were 80% (12/15 lesions) and 50% (1/2 lesions), respectively. Given the small samples, it was difficult to analyze the differences in fluorescent increase between the two gGlu-HMRG concentrations. Further investigation is needed to confirm the safety and feasibility of this technique in humans prior to the future application of this fluorescent imaging for clinical diagnosis.

In conclusion, topically spraying gGlu-HMRG enabled rapid and selective fluorescent imaging of colorectal tumors owing to the upregulated GGT activity in cancer cells.

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Disclosure Statement

The authors have no commercial association that might be a conflict of interest in relation to our submitted manuscript.

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New Image-Enhanced Endoscopy

NBI/BLI Atlas

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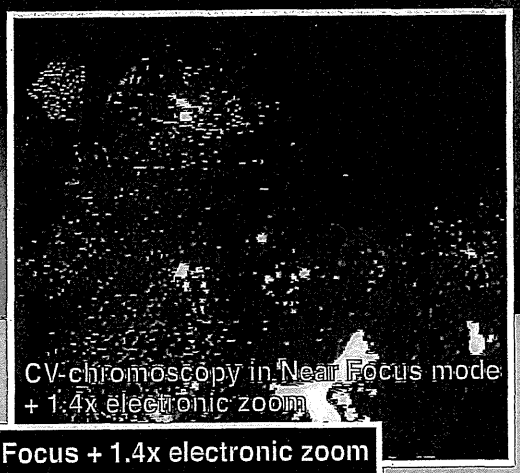

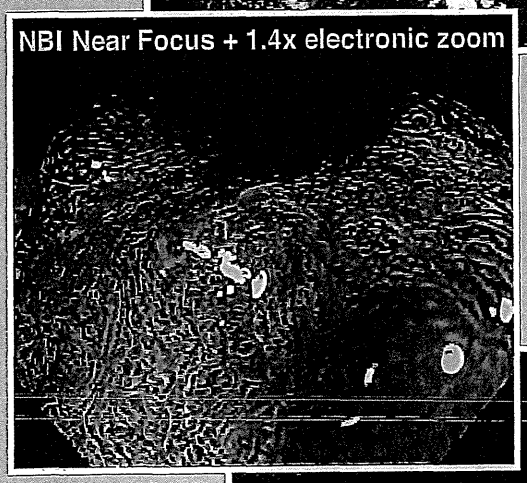

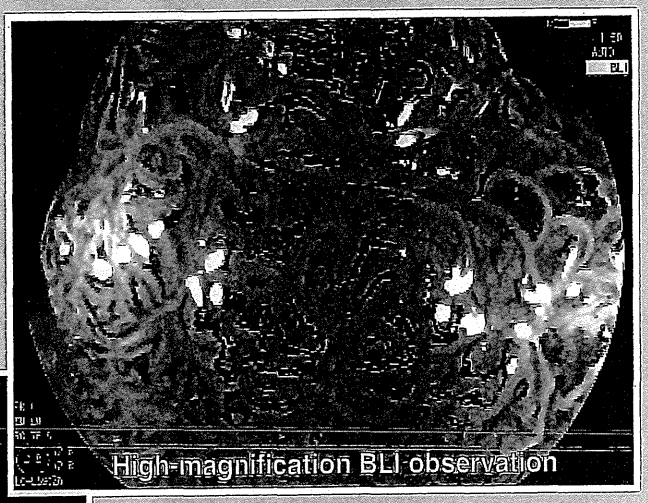

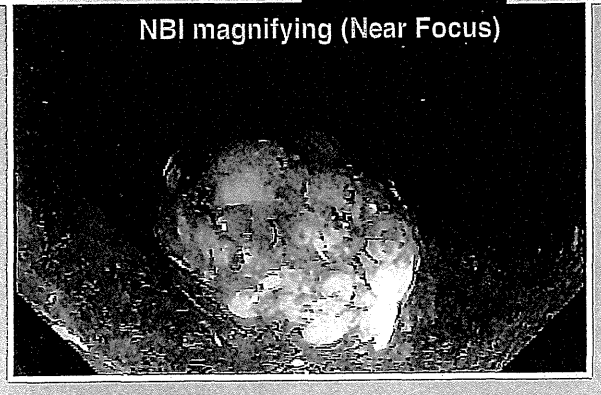

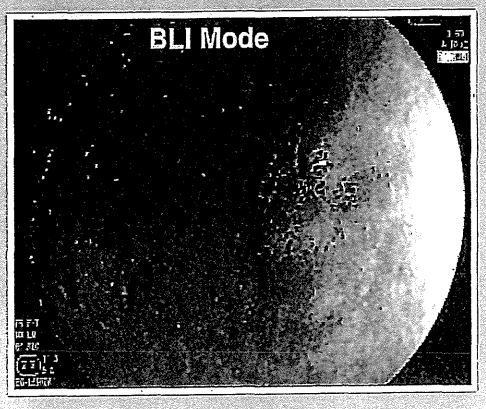

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