A

| kinase | Cetuximab による活性変動 | Activation loop |
|---|---|---|
| TAOK1 | 1.00 | adfgsasmaspan*sfvgtpy |
| TAOK2 | 1.12 | gdfgsasimapan*sfvgtpy |
| TAOK3 | 1.23 | adfgsasmaspan*sfvgtpy |
| PDPK1 | 1.12 | tdfgtakvlspeskqaran*sfvgtaq |
| SLK | 1.06 | adfgvsakntrtiqrrd*sfigtpy |
| STK10 | 0.99 | adfgvsaknlktlqkrd*sfigtpy |

B

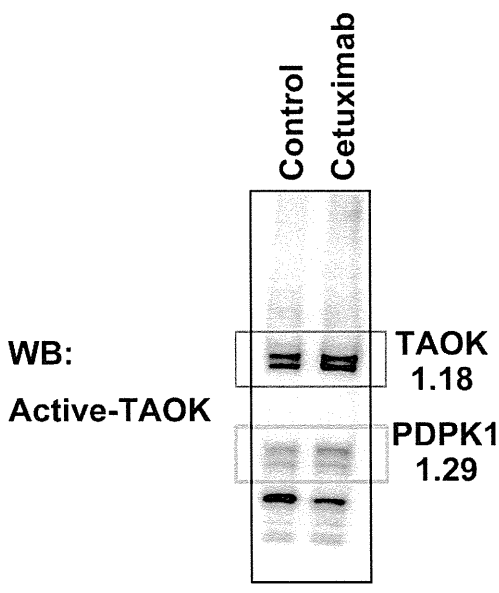


図6. 活性型TAOKの活性測定

A. セツキシマブ処理したDLD1細胞と、処理していないDLD1細胞のTAOK活性を測定した。活性型TAOK抗体を用いたアフィニティー精製を行い、精製物をLC-MS/MS解析した。TAOK1/2/3、PDPK1、SLK、STK10の活性を測定することができた。それぞれのキナーゼ毎に、セツキシマブ処理による活性変動値を示した。TAOK1/2/3、PDPK1、SLK、STK10の活性化ループ領域のアミノ酸配列を右に示した。リン酸化部位（＊）の後に続くアミノ酸配列が類似していた。

B. セツキシマブ処理したDLD1細胞と、処理していないDLD1細胞をウエスタンブロット解析した。TAOKとPDPK1と予測される位置に、バンドが検出された。

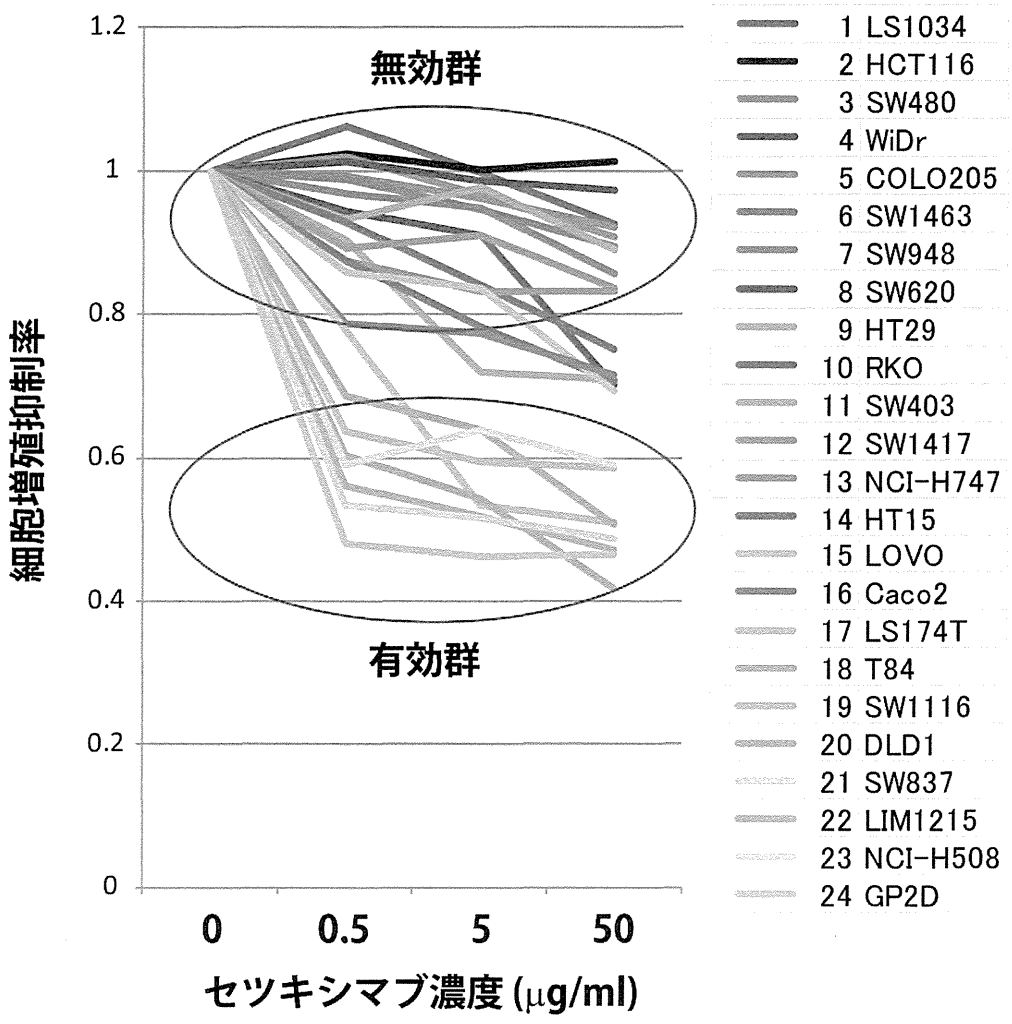


図7. 大腸癌培養細胞株のセツキシマブ感受性分類
24種の大腸癌培養細胞株をセツキシマブ（0, 0.5, 5, 50 μg/ml）で処理し、72時間後にWST-8試薬を用いて細胞増殖を調べた。セツキシマブを添加していない場合（0 μg/ml）の増殖レベルを1とした時の、増殖レベルをプロットした。

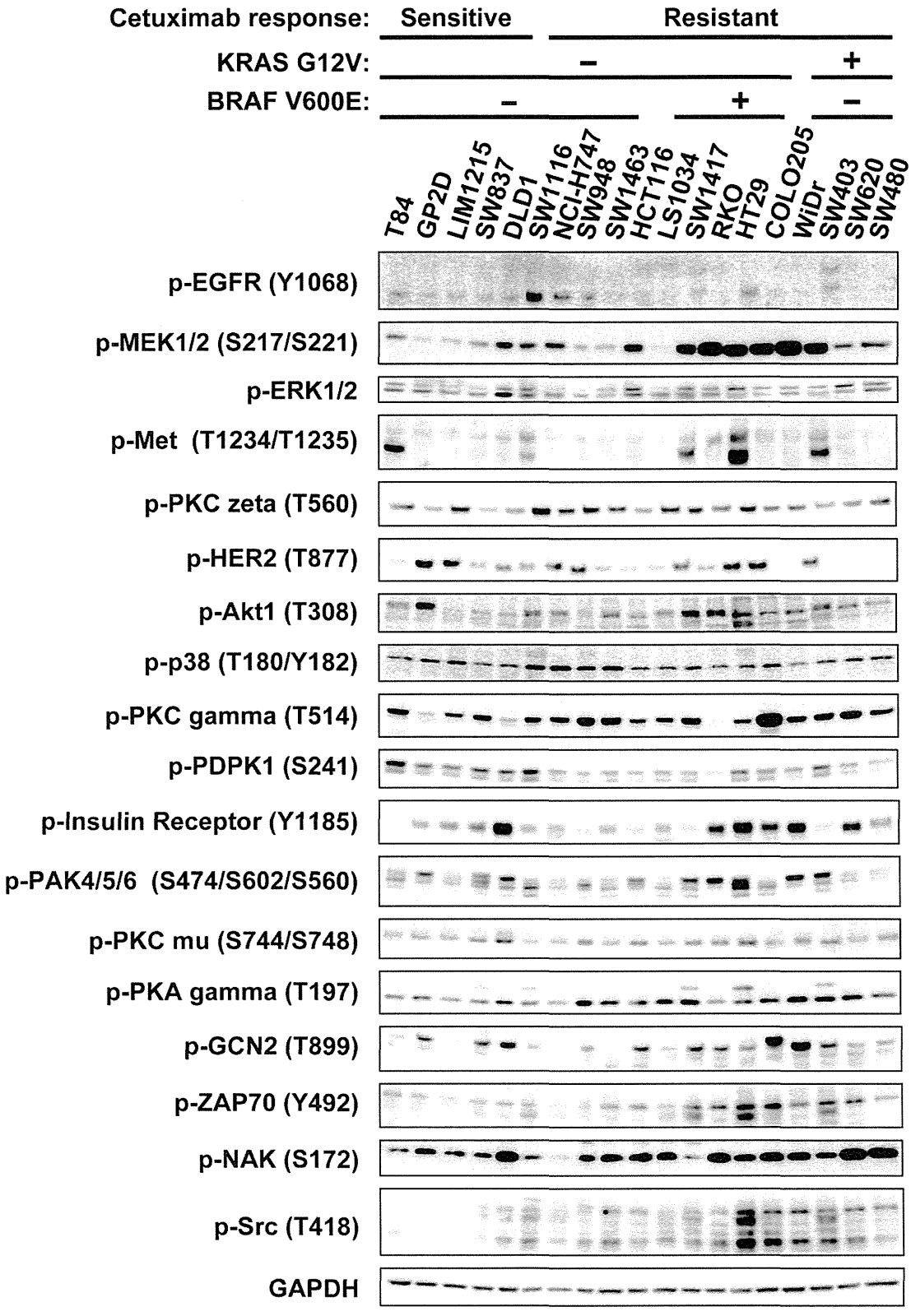


図 8. セツキシマブ耐性、感受性大腸癌細胞株のウエスタンブロット解析

各種大腸癌細胞株を活性型キナーゼ抗体を用いたウエスタンブロットで解析した。

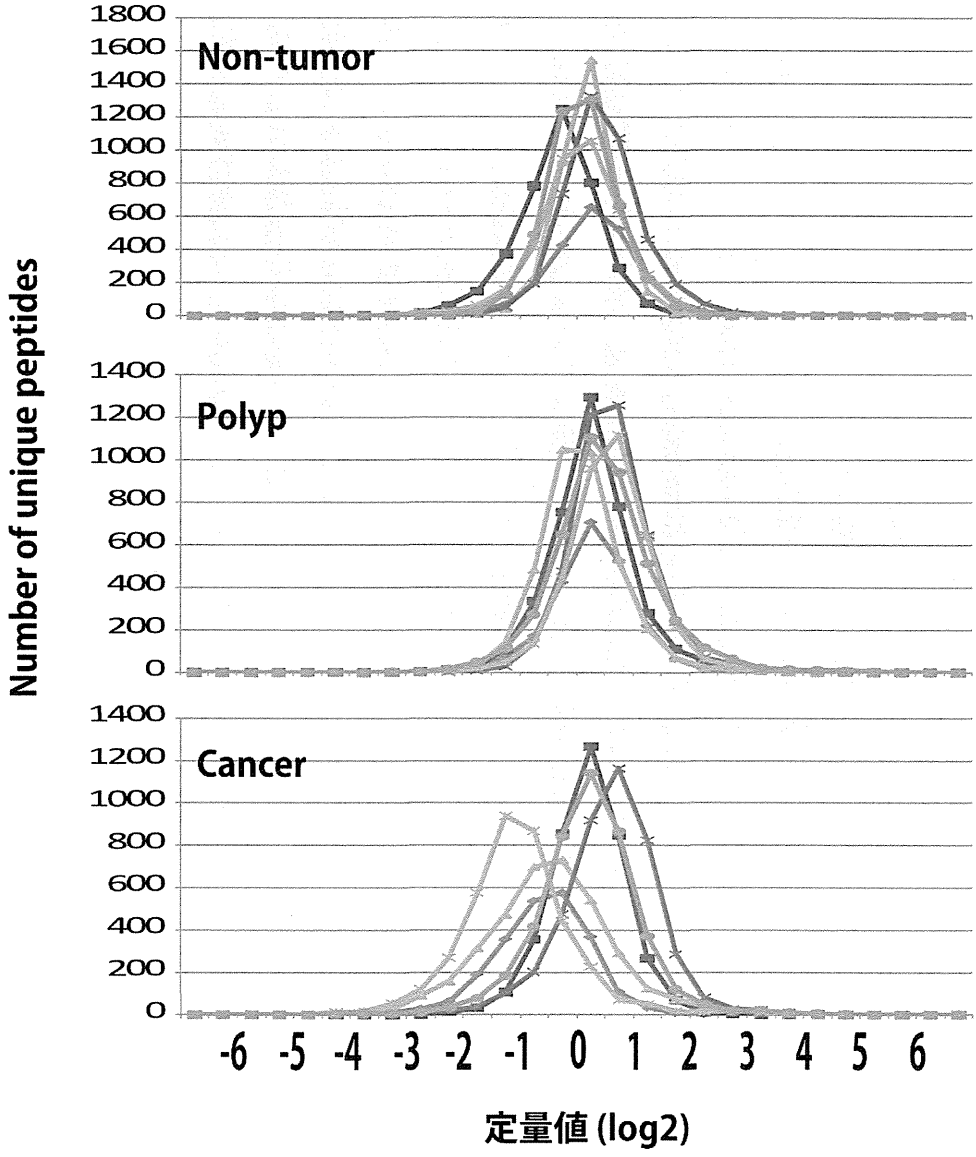


図 9. 大腸組織の大規模リン酸化プロテオーム解析

大腸癌組織（cancer）とその周辺非癌部（non-tumor）、および大腸ポリープをそれぞれ 6 検体ずつ定量的大規模リン酸化プロテオーム手法を用いて解析した。得られた 2000~3000 種類のリン酸化ペプチドの定量値の分布を各組織ごとにプロットした。

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分担研究報告書

# キナーゼ活性化レベル測定 SRM 法による抗 EGFR 抗体薬効果予測法の検証に必要な臨床検体の収集

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研究要旨
キナーゼ活性化レベル測定 SRM 法による、抗 EGFR 抗体薬効果予測法の検証に必要な臨床検体を 82 検体収集した。

## A. 研究目的

キナーゼ活性化レベル測定 SRM 法で、抗 EGFR 抗体薬の効果予測が可能かどうかを検証するために必要となる臨床検体を収集した。

## B. 研究方法

### 臨床検体の収集・保存

千葉大学医学部附属病院食道胃腸外科を受診する者のうち、検査結果から消化管腫瘍と診断された者の外科的切除標本を収集した。

タンパク質の安定性やキナーゼの活性化指標リン酸化修飾を維持すること目的に、標本は可能な限り 1～2 時間以内に凍結保存した。切除後から凍結までの間は、生理食塩水を含ませたガーゼに包んだ状態で、低温下（4℃）で保存した。

### 倫理面への配慮

＜研究対象となる個人の人権の擁護＞

研究対象患者には、人権擁護について明確に記した文書で説明を行った。特に下記の２点に配慮した。

１．試料提供に同ずるか否かは対象者の自由意思であり、試料提供しない場合でも不利益を受けない。

２．いったん同意した場合でも不利益を受けることなく、いつでも撤回することができる。

本研究は、平成 17 年４月１日施行の「ヒトゲノム・遺伝子解析研究に関する倫理指針」を尊守し、千葉大学大学院医学研究院生命倫理審査委員会で承認されたプロトコル（課題

名：消化管腫瘍における遺伝子・蛋白動態解析研究）に従って行われた。

＜対象者に理解を求め同意を得る方法＞

患者本人に対し文書を用いて以下の項目を説明し、自由意思に基づく文書による同意を受けた。

１．研究概要
２．研究の意義・目的
３．研究の方法
４．費用について
５．個人情報の保護
６．予測される結果について、遺伝子解析結果・研究計画の開示について
７．研究成果の公表について
８．試料などの他の研究への利用について
９．研究協力することによる利益と不利益
１０．　遺伝カウンセリングについて
１１．　問い合わせ等の連絡先

＜個人情報の保護の方法＞

患者の個人情報が外部に洩れることのないように厳重に管理した。試料は個人情報管理者及び分担管理者を設け、連結可能匿名化することにより研究者に患者の特定ができないようにした。

## C. 研究結果

約一年間の間に 82 症例の大腸癌切除標本（癌部および周辺非癌部組織）を収集した。そのうち、23 検体は 1 時間以内に凍結保存した。抗 EGFR 抗体薬であるセツキシマブもしくはパニツムマブが投与された症例は 3 例であった。また、本研究においては、報告すべき倫理的問題事象は発生しなかった。

## D. 結論

昨年度に収集した 70 検体と合わせると、2 年間で収集した検体数は 152 となった。そのうち、切除後 1 時間以内に凍結保存したものは 61 検体であり、抗 EGFR 抗体薬投薬症例は 9 例であった。これらの検体を用いることで、キナーゼ活性化レベル測定による、抗 EGFR 抗体薬効果予測法の初期的検証を行う事ができる。

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## F. 知的財産権の出願・登録状況

＜公開＞

1. 発明の名称：リポソーム複合体.
   発明者：岡本芳晴、田村裕、菅波晃子、林秀樹、真殿智行、松原久裕、豊田太郎.
   国際公開日：平成２５年４月１１日
   国際公開番号：WO2013/051732
2. 発明の名称：近赤外線の波長特性を利用した非侵襲性医療装置
   発明者：岡本芳晴、田村裕、菅波晃子、豊田太郎、林秀樹、真殿智行、松原久裕
   公開日：平成 25 年 11 月 14 日
   公開番号：特開 2013-230211
3. 発明の名称：内視鏡用の穿刺針（届出時：内視鏡下ドリル式組織採取針）
   発明者：上里昌也、 松原久裕
   出願日：平成 25 年 11 月 1 日
   出願番号：特願 2013-228683 号

研究成果の刊行に関する一覧表

雑誌（関連が強いものの一部）

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
|---|---|---|---|---|---|
| 久家貴寿<br>足立淳<br>朝長毅 他 | Identification of Missing Proteins in the neXtProt Database and Unregistered Phosphopeptides in the PhosphoSitePlus Database As Part of the Chromosome-Centric Human Proteome Project. | J Proteome Res | 12 | 2414-2421 | 2013 |

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# Identification of Missing Proteins in the neXtProt Database and Unregistered Phosphopeptides in the PhosphoSitePlus Database As Part of the Chromosome-Centric Human Proteome Project

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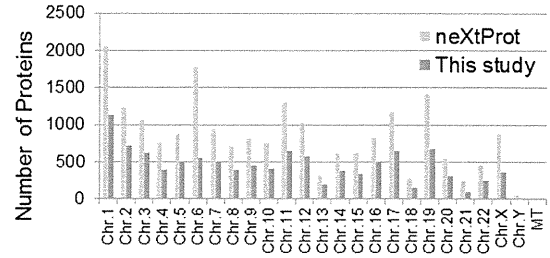
Supporting Information

ABSTRACT: The Chromosome-Centric Human Proteome Project (C-HPP) is an international effort for creating an annotated proteomic catalog for each chromosome. The first step of the C-HPP project is to find evidence of expression of all proteins encoded on each chromosome. C-HPP also prioritizes particular protein subsets, such as those with post-translational modifications (PTMs) and those found in low abundance. As participants in C-HPP, we integrated proteomic and phosphoproteomic analysis results from chromosome-independent biomarker discovery research to create a chromosome-based list of proteins and phosphorylation sites. Data were integrated from five independent colorectal cancer (CRC) samples (three types of clinical tissue and two types of cell lines) and lead to the identification of 11,278 proteins, including 8,305 phosphoproteins and 28,205 phosphorylation sites; all of these were categorized on a chromosome-by-chromosome basis. In total, 3,033 "missing proteins", i.e., proteins that currently lack evidence by mass spectrometry, in the neXtProt database and 12,852 unknown phosphorylation sites not registered in the PhosphoSitePlus database were identified. Our in-depth phosphoproteomic study represents a significant contribution to C-HPP. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium with the data set identifier PXD000089




KEYWORDS: *Chromosome-Centric Human Proteome Project, missing protein, phosphopeptide, IMAC, colorectal cancer, FASP, neXtProt, PhosphoSitePlus*

## INTRODUCTION

The Chromosome-Centric Human Proteome Project (C-HPP) is a worldwide effort by proteomics researchers to create expression profiles of the approximately 20,000 genes encoded on all human chromosomes and build a database.[1] Protein expression patterns are closely associated with the location of the gene on a chromosome and are correlated with diseases associated with chromosomal abnormalities. Therefore, a comprehensive understanding of the protein expression profile of each chromosome is critical for biological studies and clinical research. The initial aim of C-HPP was to identify at least one protein isoform for every gene encoded by the human genome. Proteins not detected by antibody or proteomic analysis using mass spectrometry are called "missing proteins".[2] Currently, there are about 6,000 missing proteins among all of the proteins in the neXtProt database.[3] One reason why missing proteins are undetectable is that protein expression differs significantly between tissue and cell types. Although the number of proteins that can be identified in a single analysis has greatly increased due to recent advances in mass spectrometric techniques, complete expression profiles of all proteins will require the integration and analysis of data from a wide variety of samples.

C-HPP also aims to map specific protein variations such as post-translational modifications (PTMs), alternative splicing, and protease-processed variants.[2] Protein phosphorylation is a key regulator of cellular signal transduction processes, and its deregulation is involved in the onset and progression of various human diseases such as cancer and inflammatory and metabolic disorders.[4−7] Recent advances in proteomics, especially phosphopeptide enrichment strategies such as immobilized metal ion affinity chromatography (IMAC) and $TiO_2$ affinity chromatography,[8] have enabled the identification of up to several thousands of site-specific phosphorylation events within one large-scale analysis.[9−19]

As participants in C-HPP, we have integrated proteomic and phosphoproteomic analysis data from human colorectal cancer tissue and cell lines and created a chromosome-based list of identified proteins. Newly detected proteins and phosphorylated peptides were identified from the neXtProt and PhosphoSitePlus databases.

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## MATERIALS AND METHODS

### Tissue and Cell Culture Samples

Colorectal cancer tissue and tumor-adjacent normal tissue samples were obtained from 44 patients at Chiba University School of Medicine. Tissue samples were frozen in liquid nitrogen and stored at −80 °C until analysis. Written informed consent was obtained from each patient before surgery, and the protocol was approved by the ethics committees of the Proteome Research Center, National Institute of Biomedical Innovation, and the Chiba University School of Medicine. Cell cultures used were HCT116, SW480, and SW620. HCT116, a colorectal cancer cell line, was grown in RPMI 1640 medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and penicillin/streptomycin (Invitrogen). Cells were maintained at 37 °C in an incubator supplemented with 5% $CO_2$ until they grew to 80% confluence. SW480 and SW620, colon cancer cell lines, were grown at 37 °C and 5% $CO_2$ for at least five passages in SILAC media (R1780-RPMI-1640 without arginine, lysine, leucine (Sigma–Aldrich Corp., St. Louis, MO, USA) with 10% dialyzed fetal bovine serum (Invitrogen) and 100 U/mL penicillin/streptomycin (Invitrogen)) containing 84 mg/L L-arginine (Arg0) and 40 mg/L L-lysine (Lys0) (light), or $^{13}C_6{}^{15}N_4$-L-arginine (Arg10) and $^{13}C_6$-L-lysine (Lys6) (heavy) and 50 mg/L L-leucine.

### Protein Extraction and Digestion

Protein extraction and proteolytic digestion were performed using a filter-assisted sample preparation (FASP) protocol.[20] Tissue samples or pellets of cultured cells were homogenized by sonication in FASP buffer [1% SDS, 0.1 M DTT, in 0.1 M Tris/HCl, pH 7.6 and PhosSTOP phosphatase inhibitor cocktail (Roche, Mannheim, Germany)]. Protein concentration was determined using a DC protein assay kit (Bio-Rad, Richmond, CA, USA). A total of 10 mg (for phosphoproteomic analysis) or 100 μg (for proteomic analysis) of extracted proteins was digested using 1:100 (w/w) trypsin (proteomics grade; Roche) for 12 h at 37 °C. Digested peptides were concentrated and purified using a C18 Sep-PAK cartridge (Waters, Milford, MA, USA).

### Phosphopeptide Enrichment

Phosphopeptide enrichment was performed using immobilized Fe(III) affinity chromatography (Fe-IMAC) as described previously.[21] The Fe-IMAC resin was prepared from Probond (Nickel-Chelating Resin; Invitrogen) by substituting $Ni^{2+}$ on the resin with $Fe^{3+}$. $Ni^{2+}$ was released from Probond upon treatment with 50 mM EDTA-2Na, and then $Fe^{3+}$ was chelated to the ion-free resin upon incubation with 100 mM $FeCl_3$ in 0.1% acetic acid. The Fe-IMAC resin was packed into an open column for large-scale enrichment. Following equilibration of the resin with loading solution (60% acetonitrile/0.1% TFA), the peptide mixture was loaded onto the IMAC column. After washing with loading solution (9 times the volume of the IMAC resin) and 0.1% TFA (3 times the volume of the IMAC resin), phosphopeptides were eluted using 1% phosphoric acid (2 times the volume of the IMAC resin).

### iTRAQ Labeling

Enriched peptides were labeled with isobaric tags for relative and absolute quantification reagents (iTRAQ 4 plex; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Peptide mixtures desalted with C18 Stage-Tips were incubated in the iTRAQ reagents for 1 h. iTRAQ 115, 116, and 117 were used for labeling individual samples, and iTRAQ 114 was used as the reference sample, a mixture of aliquots of all samples. The reaction was terminated by the addition of an equal volume of distilled water. The labeled samples were combined, acidified with trifluoroacetic acid, and desalted with C18 Stage-Tips.

### Strong Cation Exchange Chromatography (SCX)

The peptides were fractionated using a HPLC system (Shimadzu Prominence UFLC) fitted with an SCX column (50 mm × 2.1 mm, 5 μm, 300 Å, ZORBAX 300SCX; Agilent Technology). The mobile phases consisted of buffer A [25% acetonitrile and 10 mM $KH_2PO_4$ (pH 3)] and B [25% acetonitrile, 10 mM $KH_2PO_4$ (pH 3), and 1 M KCl]. The labeled peptides were dissolved in 200 μL of buffer A and separated at a flow rate of 200 μL/min using a four-step linear gradient: 0% B for 30 min, 0% to 10% B in 15 min, 10% to 25% B in 10 min, 25% to 40% B in 5 min, 40% to 100% B in 5 min, and 100% B for 10 min. Fractions were collected and desalted using C18-Stage Tips (number of fractions: CRC tissues_1 peptides, 30 fractions; CRC tissues_1 phosphopeptides, 25 fractions; HCT116 peptides, 34 fractions; HCT116 phosphopeptides, 32 fractions; SW480 + SW620 peptides, 25 fractions; SW480 + SW620 phosphopeptides, 30 fractions; CRC tissues_2 non-tumor phosphopeptides, 30 fractions; CRC tissues_2 tumor phosphopeptides, 30 fractions).

### LC–MS/MS Analysis

Fractionated peptides were analyzed using an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoLC interface (AMR, Tokyo, Japan), a nanoHPLC system (Michrom Paradigm MS2) and an HTC-PAL autosampler (CTC, Analytics, Zwingen, Switzerland). The analytical column was made in-house by packing L-column2 C18 particles (Chemical Evaluation and Research Institute (CERI), Tokyo, Japan), into a self-pulled needle (200 mm length × 100 μm inner diameter). The mobile phases consisted of buffer A (0.1% formic acid and 2% acetonitrile) and B (0.1% formic acid and 90% acetonitrile). Samples dissolved in buffer A were loaded onto a trap column (0.3 × 5 mm, L-column ODS; CERI). The nanoLC gradient was delivered at 500 nL/min and consisted of a linear gradient of buffer B developed from 5% to 30% B in 180 min. A spray voltage of 2000 V was applied.

Full MS scans were performed using an orbitrap mass analyzer (scan range $m/z$ 350–1500, with 30 K fwhm resolution at $m/z$ 400). The 10 most intense precursor ions were selected for the MS/MS scans, which were performed using collision-induced dissociation (CID) and higher energy collision-induced dissociation (HCD, 7500 fwhm resolution at $m/z$ 400) for each precursor ion. The dynamic exclusion option was implemented with a repeat count of 1 and exclusion duration of 60 s. Automated gain control (AGC) was set to 1.00e + 06 for full MS, 1.00e + 04 for CID MS/MS, and 5.00e + 04 for HCD MS/MS. The normalized collision energy values were set to 35% for CID and 50% for HCD.

The CID and HCD raw spectra were extracted and searched separately against UniProtKB/Swiss-Prot (release-2010_05), which contains 20,295 sequences (the forward and reverse-decoy) of *Homo sapiens*, using Proteome Discoverer 1.3 (Thermo Fisher Scientific) and Mascot v2.3. The precursor mass tolerance was set to 7 ppm, and fragment ion mass tolerance was set to 0.5 Da for CID and 0.01 Da for HCD. The search parameters allowed two missed cleavage for trypsin,


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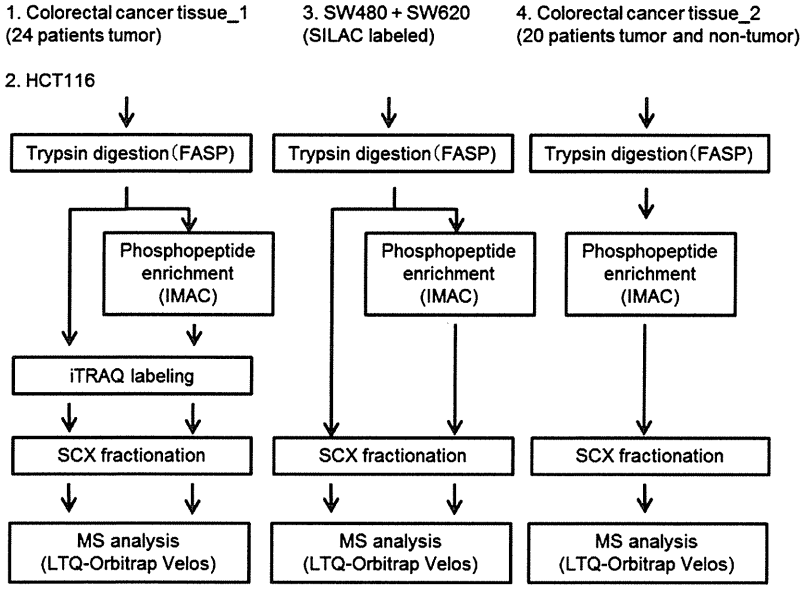


**Figure 1.** Schematic representation of the experimental work flow for the proteomic and phosphoproteomic analyses of the four experiments. SW480 + SW620: a mixture of protein extracts obtained from SW480 and SW620 cells. After trypsin digestion, each sample was separated for proteomic (100 $\mu$g) or phosphoproteomic (10 mg) analysis. Digested samples were separated by using an SCX column. LC–MS/MS, requiring 3-h runs, was performed using an LTQ-Orbitrap Velos.

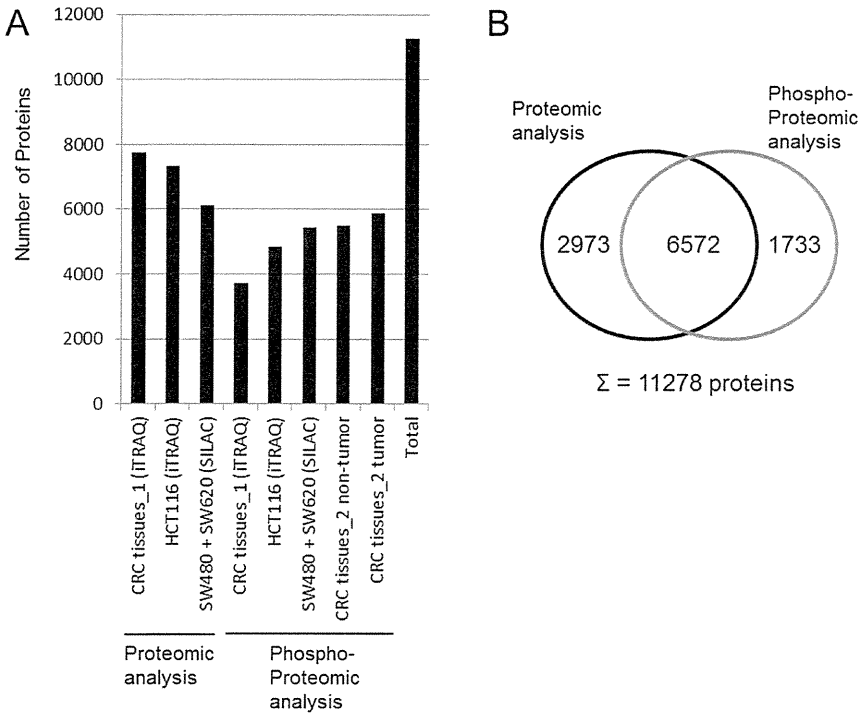


**Figure 2.** Number and overlap of identified proteins from the proteomic and phosphoproteomic analyses. (A) Number of identified proteins from the proteomic and phosphoproteomic analyses of the 8 data sets. (B) Proportion of proteins identified in each analysis and overlap between proteins identified by the proteomic and phosphoproteomic analyses.

fixed modifications (carbamidomethylation at cysteine), and variable modifications (oxidation at methionine). Fixed modifications were set for CRC tissue and HCT116 (iTRAQ labeling at lysine and the N-terminal residue) and SW480 + SW620 (SILAC labeling 13C(6) 15N(4) Arg, 13C(6) Lys). Variable modifications were added for phosphoproteomic analysis (phosphorylation at serine, threonine, and tyrosine). In the workflow of Proteome Discoverer 1.3, following the Mascot search, the phosphorylated sites on the identified peptides were assigned again using the PhosphoRS algorithm, which calculated the possibility of the phosphorylated site from the spectra matched to the identified peptides.[22] The score threshold for peptide identification was set at 1% false-discovery rate (FDR) and 75% phosphoRS site probability. FDR was calculated using the Percolator algorithm for peptide sequence analysis. Percolator uses >30 features of a peptide spectral match (PSM) to distinguish true positives from random matches.


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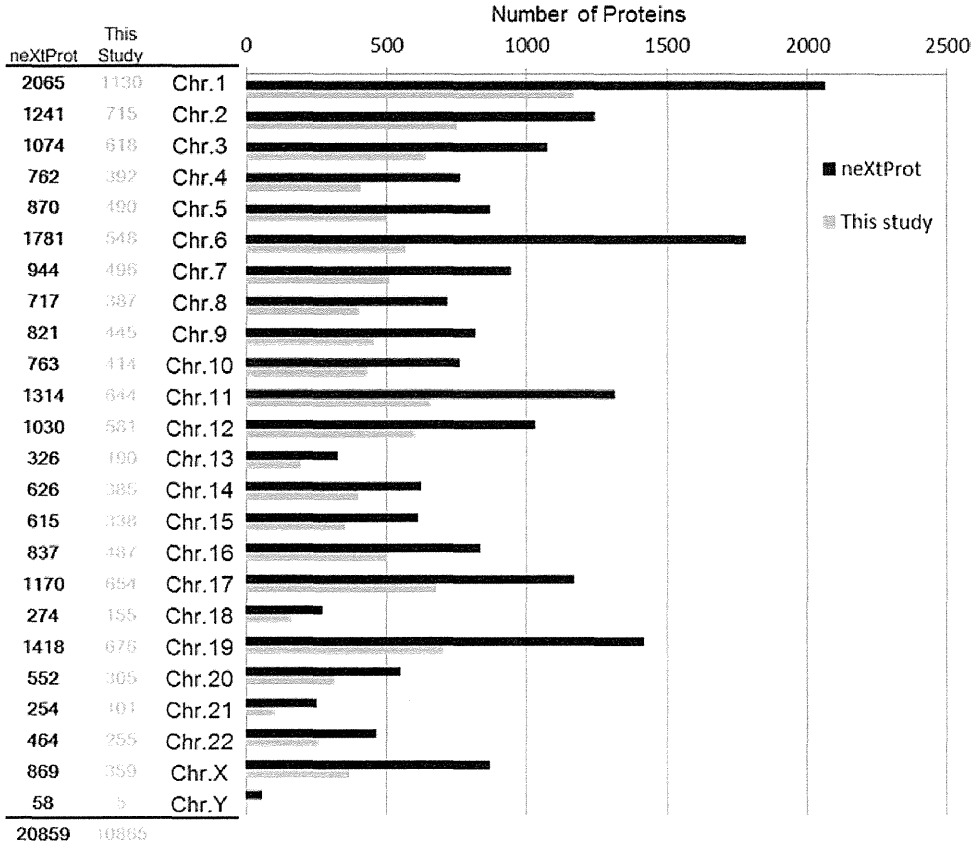


**Figure 3.** Chromosomal distribution of the identified proteins (gray) in relation to total proteins (black) registered in the neXtProt database.

### Bioinformatics Analysis

Chromosomal locations and missing protein analyses of identified proteins were elucidated using the neXtProt database (http://www.nextprot.org/db/), and identified phosphorylation sites were elucidated using the PhosphoSitePlus database (http://www.phosphosite.org/). The function of identified missing proteins was elucidated by ingenuity pathway analysis software (Ingenuity Systems, Redwood City, USA).

### Stable Isotope-Labeled Peptides

Stable isotope-labeled standard peptides (SIS peptides, crude grade) were synthesized (Thermo Fisher Scientific, Ulm, Germany). A single lysine was replaced by isotope-labeled lysine ($^{13}C_6$, 98%, $^{15}N_2$, 98%). The SIS peptides were dissolved in distilled water at a concentration of 1 $\mu$g/$\mu$L and stored at −80 °C. A mixture of these SIS peptides was added to colorectal carcinoma phosphoproteomic samples.

## RESULTS

As part of the C-HPP project, we combined the eight data sets from four different experiments obtained from colorectal cancer tissue and colon cancer cells; these experiments included three quantitative analyses and one non-quantitative analysis. Colorectal cancer tissues and colon cancer cells were first solubilized and trypsin-digested using the FASP method.[20] Phosphopeptides were then enriched using the IMAC method. These peptides and phosphopeptides were fractionated on a Strong Cation-Exchange (SCX) column before LC−MS/MS using an LTQ-Orbitrap mass spectrometer (Figure 1). Proteome Discoverer 1.3 software was used to analyze the RAW data files, Mascot was used as the search engine, and UniProtKB/Swiss-Prot (release-2010_05) was the database. Following data integration, 11,278 proteins were identified with Peptide FDR ≤1.0 containing at least one unique peptide corresponding to one protein in the database (Figure 2A, Supplementary Table 1−4). Of these, 8,305 proteins were identified as phosphorylated. Among the total identified proteins, 673 proteins were identified only with CID, and 386 proteins were identified only with HCD. Also, 4924 phosphopeptides were identified only with CID, and 3538 phosphopeptides were identified only with HCD. A total of 6,572 proteins were commonly identified in the proteomic and phosphoproteomic analyses (Figure 2B). However, a proportion of proteins were found not to overlap in the analyses. This is probably due to the abundance and complexity of the proteins and phosphoproteins in the samples, which prevent proteomics and phosphoproteomics to identify all of the proteins and phosphoproteins present.

Quantitative analyses were performed to investigate the differences between metastatic and non-metastatic cases by using clinical tissue and two types of cultured cells (a mixture of SW620 + SW480 and HCT116 cells). Clinical tissue samples of primary colorectal cancer obtained from 12 patients with or without metastasis were pooled. Cancers without metastasis were labeled with iTRAQ 114 or 116, and those with metastasis were labeled with iTRAQ 115 or 117. We also performed quantitative analyses between metastatic and non-metastatic cell lines. HCT116 metastatic clone was established by orthotopic implantation model mouse, and its protein expression was compared with that of the parent clone. SW620 cell line is a lymph node metastatic variant of SW480. HCT116 parent clone was labeled with iTRAQ 114 or 115, and metastatic clone was labeled with iTRAQ 116 or 117. SW480 and SW620 were reciprocally labeled with light and heavy


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**Table 1. Number of Identified Proteins in Each Chromosome**

| | | | proteomic analysis | | | phosphoproteomic analysis | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | neXtProt | total | CRC tissue_1 | HCT116 | SW480 + SW620 | CRC tissue_1 | HCT116 | SW480 + SW620 | CRC tissue_2 non-tumor | CRC tissue_2 tumor |
| Chr.1 | 2065 | 1171 | 808 | 767 | 611 | 356 | 494 | 554 | 568 | 598 |
| Chr.2 | 1241 | 753 | 516 | 481 | 416 | 259 | 305 | 378 | 371 | 414 |
| Chr.3 | 1074 | 642 | 445 | 425 | 360 | 209 | 275 | 310 | 321 | 341 |
| Chr.4 | 762 | 412 | 267 | 251 | 185 | 126 | 178 | 176 | 210 | 224 |
| Chr.5 | 870 | 508 | 339 | 319 | 285 | 168 | 210 | 249 | 255 | 281 |
| Chr.6 | 1781 | 569 | 395 | 347 | 302 | 186 | 233 | 260 | 272 | 291 |
| Chr.7 | 944 | 511 | 364 | 319 | 305 | 160 | 204 | 269 | 244 | 253 |
| Chr.8 | 717 | 402 | 270 | 263 | 196 | 125 | 177 | 188 | 176 | 199 |
| Chr.9 | 821 | 458 | 312 | 294 | 229 | 161 | 197 | 212 | 230 | 225 |
| Chr.10 | 763 | 434 | 298 | 300 | 254 | 131 | 186 | 223 | 232 | 245 |
| Chr.11 | 1314 | 660 | 477 | 447 | 388 | 227 | 299 | 340 | 352 | 360 |
| Chr.12 | 1030 | 603 | 399 | 409 | 330 | 207 | 274 | 296 | 294 | 316 |
| Chr.13 | 326 | 194 | 140 | 127 | 114 | 62 | 80 | 97 | 87 | 99 |
| Chr.14 | 626 | 400 | 283 | 252 | 218 | 132 | 166 | 180 | 203 | 210 |
| Chr.15 | 615 | 353 | 237 | 234 | 181 | 121 | 163 | 184 | 174 | 197 |
| Chr.16 | 837 | 508 | 341 | 333 | 260 | 159 | 230 | 245 | 236 | 258 |
| Chr.17 | 1170 | 678 | 437 | 484 | 399 | 239 | 330 | 339 | 347 | 361 |
| Chr.18 | 274 | 161 | 102 | 112 | 74 | 50 | 67 | 64 | 70 | 77 |
| Chr.19 | 1418 | 707 | 479 | 471 | 362 | 253 | 336 | 332 | 341 | 369 |
| Chr.20 | 552 | 313 | 223 | 194 | 182 | 119 | 137 | 151 | 138 | 160 |
| Chr.21 | 254 | 103 | 78 | 68 | 64 | 36 | 41 | 51 | 47 | 53 |
| Chr.22 | 464 | 262 | 187 | 182 | 153 | 93 | 109 | 123 | 129 | 130 |
| Chr.X | 869 | 369 | 261 | 222 | 222 | 120 | 144 | 189 | 175 | 188 |
| Chr.Y | 58 | 6 | 4 | 2 | 1 | 1 | 0 | 0 | 1 | 2 |
| NA[a] | | 101 | 73 | 30 | 17 | 11 | 11 | 17 | 18 | 16 |
| total | 20845 | 11278 | 7735 | 7333 | 6108 | 3355 | 4352 | 5427 | 5491 | 5867 |

[a]Not applicable in neXtProt.

stable isotope amino acids (lysine and arginine). HCT116 has a mutation in codon 13 of the ras protooncogene, while SW480 and SW620 have a mutation in codon 12. Among 8305 proteins and 28,205 phosphopeptides, 472 proteins and 2547 phosphopeptides showed >2-fold differences between metastatic and non-metastatic tissues and cell lines (either upregulated or downregulated).

A total of 20,845 proteins have been registered in the neXtProt database. Proteins identified in this study were referred to the database and accounted for 53.6% (11,177/20,845) of all the proteins registered in the neXtProt database; their chromosomal locations are shown in Figure 3 and Table 1. Of the proteins registered in the neXtProt database, the expression of 14,612 proteins (70.1% of the total of 20,845 proteins) has been confirmed by mass spectrometry or antibody assay (protein level 'yes'), whereas 10,649 proteins (51.1% of the total of 20,845 proteins) have been identified only by MS analysis (proteomic level 'yes') (Table. 2). Cross-checking the 11,278 proteins identified in this study with the neXtProt database revealed 1,145 proteins currently lacking evidence of protein expression by mass spectrometry or antibody assays, and 3,033 proteins lacking evidence by mass spectrometry. These "missing proteins (protein level = no and proteomic level = no)" are listed on a chromosome-by-chromosome basis (Figure 4).

**Table 2. Number of Proteins Identified at the Protein or Proteomic Level**

| evidence | | neXtProt | this study |
|---|---|---|---|
| protein level | yes | 14612 | 10032 |
| | no | 6233 | 1145 |
| proteomics level | yes | 10649 | 8144 |
| | no | 10196 | 3033 |

In contrast, 28,205 phosphorylation sites were identified (Supplementary Table 5). When these phosphopeptides were cross-checked with the PhosphoSitePlus database, 15,353 registered phosphorylation sites were identified, or 12.2% of all registered sites in PhosphoSitePlus (15,353/125,433). Of these, 12,852 sites were not registered in PhosphoSitePlus (Figure 5A). The chromosomal locations of these phosphorylation sites are shown in Figure 5B. In order to verify the accuracy of the identified phosphopeptides, lysine at the C-terminus of two peptides (LYNSEESRPYTNK, SASQSSLDKLDQELK) was labeled by stable isotope ($^{13}C_6$, $^{15}N_2$). The SIS peptides were added to the extract of colorectal cancer tissue, and annotated mass spectra and extracted ion chromatogram data of SIS peptides were compared to those of nonlabeled endogenous peptides (Supplementary Figure 1).

Non-quantitative analyses were also performed using pooled colorectal carcinoma tissues and tumor-adjacent normal tissues 5−10 cm remote from the tumor. To investigate the association between phosphoproteins and biological function, gene ontology analysis was performed by using Ingenuity Pathway Analysis (IPA) software. Specifically identified phosphoproteins in normal (636 proteins) and carcinoma tissues (1020 proteins) were also analyzed by IPA. Molecular functions involved in cell cycle (normal = 9 proteins, tumor = 132 proteins; $p < 0.01$ Fisher's exact test) and DNA replication (normal = 15 proteins, tumor = 106 proteins ; $p < 0.01$)


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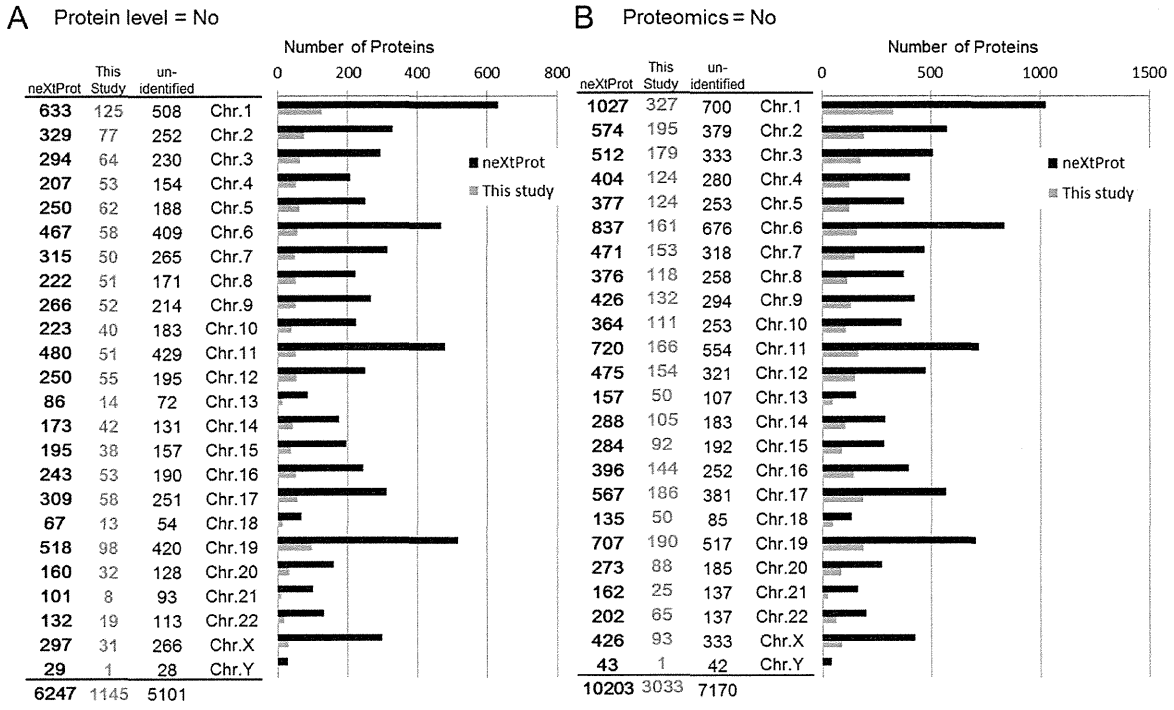


**Figure 4.** Chromosomal distribution of the identified proteins (gray) and total registered proteins (black) in the neXtProt database with no evidence of expression at the protein level (A) and at the proteomic level (B).

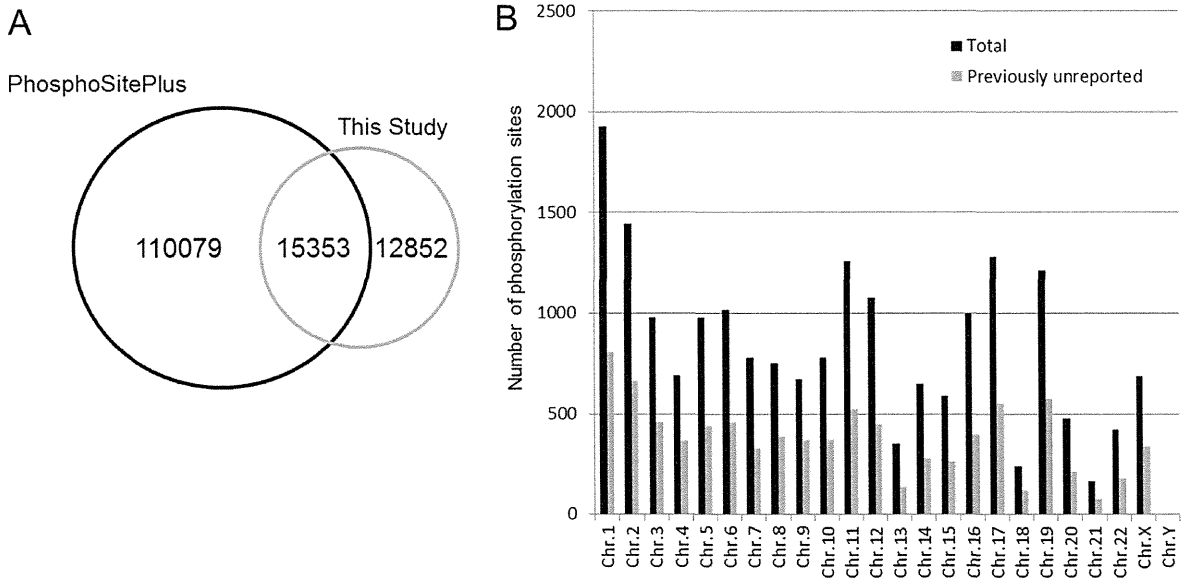


**Figure 5.** (A) Overlap between phosphorylation sites identified in this study and those registered in the PhosphoSitePlus database. (B) Chromosomal distribution of the identified previously unreported phosphorylation sites (gray) and total registered sites (black).

functions were more abundant in cancer tissues than in normal tissues (Supplementary Figure 2).

## DISCUSSION

The objective of C-HPP is to map and annotate all protein-coding genes on each human chromosome. C-HPP also prioritizes particular protein subsets such as post-translational modifications (PTMs) and low-abundance proteins. Thus, we have integrated proteomic and phosphoproteomic data obtained from a shotgun analysis using human cancer tissue and cell lines prepared for various purposes. We have integrated quantitative and non-quantitative data; quantitative analysis was performed for the relative quantification between metastatic and non-metastatic colorectal carcinoma samples, while non-quantitative analysis was performed to compare the tumor and normal tissues. As a result, we identified 11,278 proteins, 8,305 phosphoproteins, and 28,205 phosphorylation sites, and their chromosomal locations were defined using the neXtProt database. Furthermore, we were able to identify 3,033 missing proteins that currently lack evidence by mass spectrometry and 12,852 unknown phosphorylation sites that are not in the PhosphoSitePlus database.

Currently, the research group with the most advanced mass analysis system can identify over 10,000 proteins in a single


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analysis run and identify about 50% of the proteins in their comprehensive analyses using multiple cell lines.[23] Additionally, the number of phosphorylation sites identified has exponentially increased,[24] largely due to improvements in phosphopeptide enrichment methods such as IMAC[15] and $TiO_2$ affinity chromatography.[8] A phosphoproteomic study of HeLa cells identified more than 65,000 phosphopeptides using a combination of phosphopeptide enrichment and SCX chromatography.[25] Several phosphoproteomic studies using tissue samples have been reported and have identified 5,195 phosphopeptides from human dorsolateral prefrontal cortex[26] and 5,698 phosphorylation sites from tumor tissues of melanoma model mice.[27] In our study, we identified 11,278 proteins and 28,205 phosphorylation sites; some had been identified in previous reports, but a number of the proteins and phosphorylation sites are not listed in the neXtProt or PhosphoSitePlus databases. Since mass analysis systems are rapidly becoming more powerful, in the future an individual research group may be able to identify all the proteins in the human genome in one analysis. However, in order to build an extensively annotated proteome database, which is one purpose of the C-HPP project, it is necessary to combine the analyzed data of various samples from many research groups.

Our analysis increased the number of identified proteins by combining the results of proteome analysis and phosphoproteome analysis on identical samples. Even using commonly studied cell lines, combining the results of post-translational modification analysis and analysis of fractionated samples will increase the number of identified missing proteins. The data presented here are based on relative quantification, and thus to confirm protein expression and examine protein abundance and localization, validation using antibodies or selected reaction monitoring (SRM) is required. Such validations will benefit from information on the identified cell line, sample preparation methods, MS analysis data, and the sequences of the identified peptides/phosphopeptides. We and other researchers, including Muraoka and colleagues,[28] Narumi and colleagues (unpublished data), and Kume and colleagues (unpublished data), are currently using a strategy for large-scale proteomics and SRM-based validation to discover biomarkers for various diseases and aim to obtain additional proteomics data by SRM validation and quantitation that will be integrated into the C-HPP project.

## ■ ASSOCIATED CONTENT

### Supporting Information

Annotated mass spectra and retention time data from liquid chromatography; results of phosphoproteomic analysis in normal and carcinoma tissues; lists of identified proteins and peptides by phosphoproteomic and proteomic analysis; list of identified phosphorylation sites. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[29] with the data set identifier PXD000089. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

C-HPP, Chromosome-Centric Human Proteome Project; CRC, colorectal cancer; PTMs, post-translational modifications; IMAC, immobilized metal ion affinity chromatography; FASP, filter-assisted sample preparation; SCX, strong cation-exchange; FDR, false discovery rate; SRM, selected reaction monitoring; CID, collision-induced dissociation; HCD, higher energy collision-induced dissociation; LC−MS/MS, liquid chromatography tandem mass spectrometry; CE, collision energy; LTQ, linear ion trap; fwhm, full wide at half-maximum

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